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THE UNIVERSITY OF ALBERTA

Preparation of ^{67}Ga-Fibrinogen using Deferoxamine, a Bifunctional Chelating Agent

by

©

Ingrid Louise Koslowsky

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

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Faculty of Pharmacy and Pharmaceutical Sciences

EDMONTON, ALBERTA

SPRING 1987

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....

J.E. Coates ....

Date February 19, 1987

To Jack

Abstract

Various parameters which could influence the radiolabeling of human fibrinogen with ^{67}Ga were studied. Since it is difficult to directly complex radiometals such as ^{67}Ga with proteins, a bifunctional chelating agent, deferoxamine, was coupled to fibrinogen via a two-step glutaraldehyde coupling method. Specific reaction conditions, such as protein concentration, and the ratio of chelating agent to protein, were varied to determine the optimum conditions needed for maximum ^{67}Ga-fibrinogen yield with minimal loss of protein clottability. Upon completion of the coupling reaction, the protein complex was purified by gel filtration and radiolabeled with ^{67}Ga. At a mean conjugation level of 7.1 moles of deferoxamine per mole of fibrinogen, the radiolabeling efficiency averaged 42.3%, with a mean protein clottability of 62.6% and an isotopic clottability of 70.2%. Decreasing the molar ratio increased both the isotopic clottability and protein clottability; however the radiolabeling efficiency was markedly decreased. In contrast, increasing the molar ratio resulted in a noticeable increase in the radiolabeling yield at the expense of protein clottability. Increasing the protein concentration, while maintaining a constant deferoxamine-fibrinogen molar ratio, produced a marked decrease in all three parameters. Polymerization of the protein complex was observed when employing high concentrations of protein and deferoxamine-glutaraldehyde. This may have been due to inter- and intramolecular crosslinking caused by free glutaraldehyde in the solution, and may account for the decrease observed in protein clottability.

An increase in the amount of ^{67}Ga-citrate added to deferoxamine-fibrinogen did not increase the specific activity of ^{67}Ga-fibrinogen. The specific activity remained between 2 and 3 MBq/mg which was considered to be too low for clinical application as a diagnostic agent for deep vein thrombosis. Neutron activation analysis was performed on aliquots of several decayed ^{67}Ga-citrate samples to determine if the samples were contaminated with trace metals. Significant levels of zinc metal were detected in all the samples. Thus, zinc metal contamination may have contributed to the observed low yields of ^{67}Ga-fibrinogen by competing with ^{67}Ga binding sites on the deferoxamine-fibrinogen complex.

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The work described in this thesis could not have been accomplished without helpful contributions from the author's supervisors, John Scott and Dr. Alec Shysh. Their guidance and encouragement throughout the course of this work is greatly appreciated.

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I. INTRODUCTION

Deep vein thrombosis (DVT) is a common, largely preventable disorder which may lead to serious and sometimes lethal pulmonary emboli, although it often is remarkably subtle in its clinical manifestations. Indeed, clinical symptoms are evident in only approximately 50% of the cases (1). Particularly at risk are patients with myocardial infarction, cerebrovascular disease, hip fracture, and many persons following major surgery. Since the diagnostic accuracy of DVT on the basis of the physical examination and history is poor (2-6), a plethora of objective diagnostic tests have emerged in order to assist the physician when thrombophlebitis is suspected.

Contrast venography is generally recognized as the reference diagnostic test for the presence of DVT (7,8). However, the test may cause discomfort in an acutely ill patient and is not suitable for repeated examinations (9). Complications include pain, dislodgement of thrombi, extravasation of the chemicals, induced thrombosis, and allergic and adverse effects of the contrast material (10-14). In addition, the interpretation of the radiographs may be difficult and inconclusive (15) with 10 to 30 percent of the venograms failing to visualize some segment of the venous system (16). Therefore efforts have been made to develop alternatives to venography.

The noninvasive tests, Doppler ultrasound, thermography, and impedance plethysmography, all require skilled personnel in order to obtain reliable results. To date, impedance plethysmography is the only noninvasive procedure which has been vigorously evaluated (17). In addition, there is no concensus whether these techniques should be employed either singly or in combination although none have proved as reliable as the venogram when used alone (9,18).

A variety of radiopharmaceuticals have been investigated as alternatives to the above procedures for the diagnosis of deep vein thrombosis, some of which remained limited to animal experiments while others were examined in humans. Among these methods were: ^{111}In-labeled platelets (19-23), ^{99m}Tc-plasmin (24-26), radiolabeled streptokinase and urokinase (27-30), ^{99m}Tc-labeled red cells (15,31,32), ^{99m}Tc-macroaggregated albumin

(33-35), ^{99m}Tc-heparin (36,37), radiolabeled fibrinogen (38-42), radioiodinated fibrin fragments (43), radioiodinated plasminogen (44), and radiolabeled monoclonal antibodies to fibrinogen, fibrin, or platelets (45-47). However, following the preliminary evaluation of these radiopharmaceuticals in both experimental and clinical settings, few have become established in routine diagnostic practice. This is largely due to lengthy pre-imaging radiolabeling procedures, and a lack of sensitivity and/or specificity of the diagnostic techniques.

The ^{125}I-fibrinogen uptake test (FUT) (48) is one of the few diagnostic procedures which has been used extensively in clinical practice. Unfortunately, due to the emission of low energy gamma radiation, the FUT is unable to detect venous thrombosis forming in the pelvis and upper thigh. Fibrinogen has also been labeled with ^{131}I and ^{123}I, however the radiation dose from ^{131}I, and the cost of ^{123}I have precluded their widespread use.

^{99m}Tc-human fibrinogen has been prepared and studied by several investigators (49,50). Due to instability of the technetium-protein complex (51), and the lack of its sensitivity and specificity in clinical studies (18), ^{99m}Tc-labeled fibrinogen is also not an ideal radiopharmaceutical for diagnosing deep vein thrombosis.

In general, 'direct' labeling of fibrinogen and other proteins with various radiometals such as ^{99m}Tc, ^{67}Ga, and ^{111}In does not result in radiopharmaceuticals with adequate stability either *in vitro* or *in vivo*. One approach to attempt to improve the stability of a protein radiometal complex is to employ bifunctional chelating agents. This concept was first introduced into radiopharmaceutical studies by Sundberg *et al* (52). Various coupling methods and chelating agents have been investigated, many of which led to compounds which produced a high labeling yield and superior *in vivo* stability when compared with directly labeled proteins (53-55).

Among potentially applicable bifunctional chelating agents, deferoxamine, a well-known iron chelating agent, has been studied. Deferoxamine forms a 1:1 complex with trivalent metal ions (56,57) such as Fe^{3+}, Ga^{3+}, and In^{3+}, and has a free amino group which is not involved in metal binding (58). With ferric ions, deferoxamine forms complexes of high stability (log K=30.6) (59). The relative stability of Ga-deferoxamine appears to be even higher than that of ferrioxamine (60). These features make deferoxamine an attractive agent in the preparation of radiopharmaceuticals.

Therefore, the aim of this project was to establish a procedure for the routine preparation of ^{67}Ga-labeled fibrinogen by initially preparing a deferoxamine-fibrinogen conjugate which could subsequently be radiolabeled with ^{67}Ga. Two purification methods, namely dialysis and gel filtration, were compared to study the effectiveness of each in the removal of unreacted reagents from the deferoxamine-protein complex. The influence of several preparatory parameters on the clottability of deferoxamine-fibrinogen and radiolabeling efficiency of ^{67}Ga-fibrinogen were also examined in order to determine the optimum conjugation level and protein concentration of the product. To ascertain whether ^{67}Ga-fibrinogen could be useful for clinical purposes, the amount of radiogallium added to deferoxamine-fibrinogen was increased in an attempt to increase the product's specific activity. Finally, aliquots of decayed ^{67}Ga-citrate were analyzed by neutron activation analysis to determine whether extraneous metal ions were present in the radiogallium solutions, these metals possibly affecting the radiolabeling yield of ^{67}Ga-fibrinogen.

II. LITERATURE SURVEY

A variety of radioisotopes and techniques have been utilized in the past 20 to 30 years to assist with the diagnosis of venous thrombosis. These radioisotopic procedures generally fall into two broad categories (61):

1. those methods which identify the thrombus by being incorporated into the thrombus during clot formation or dissolution, and
2. those methods which entail dynamic imaging of tracer distribution.

Table 1 illustrates the many different radiopharmaceuticals which have been investigated and the method of localization or detection of the thrombi. A survey of several of these radiopharmaceuticals has been prepared by Knight (118). To date, there has been no product which can detect thrombi during their entire cycle of thrombogenesis, stabilization, and fibrinolysis. For example, radiolabeled fibrinogen is incorporated during the dynamic process of thrombus formation, whereas plasminogen and urokinase are useful in detecting old, lysing thrombi (119). Platelets are also incorporated during thrombogenesis; however, labeled platelets may be more useful in detecting arterial thrombus formation since these thrombi are composed predominantly of platelets, as opposed to venous thrombi which mainly consist of fibrinogen/fibrin (1).

Very few of the radiopharmaceuticals tabulated in Table 1 have been used clinically. Radioisotope venography employing ^{99m}Tc-macroaggregated albumin (MAA), blood pool imaging using ^{99m}Tc-red blood cells (RBC), and ^{111}In-labeled platelets (61) have all been used successfully for the diagnosis of deep vein thrombosis. However, either the sensitivity or specificity of these products usually requires another diagnostic technique to be employed, such as impedance plethysmography or contrast venography.

Radiolabeled fibrinogen, especially ^{125}I-fibrinogen, has been used extensively in order to identify those patients at risk for developing venous thrombosis (61). It is also useful in following the natural history of this condition, and to determine the effectiveness of a variety of prophylactic measures (48,120-122).

Table 1

Radioisotopic Diagnostic Techniques for Detection of Venous Thrombosis

Diagnostic Agent	Label	Proposed Mechanism of Thrombus Detection	References
Fibrinogen	^{125}I, ^{131}I, ^{123}I, ^{77}Br, ^{197}Hg, ^{111}In, ^{99m}Tc, ^{67}Ga	incorporation into thrombus during thrombogenesis	38-42,48-51,62-78
Heparin	^{99m}Tc	binds to Antithrombin III which increases binding affinity to thrombin	36,37,79,80
Platelets	^{111}In, ^{99m}Tc, ^{51}Cr	incorporation into thrombus during thrombogenesis; adhesion or adsorption onto thrombi surfaces	9,19-23,81-84
Soluble Fibrin Fibrin des-AA Fibrin des-AABB	^{125}I, ^{131}I	incorporation onto forming and stabilized thrombi	85-87
Fragment E_1 Fragment E(1,2)	^{125}I, ^{123}I, ^{131}I, ^{67}Ga	dimeric molecules derived from plasmic digestion of cross-linked fibrin; binds only to fibrin polymers	43,88,89
Fibronectin	^{125}I	high molecular weight glycoprotein with affinity for fibrin	90
Plasmin	^{99m}Tc	binds to fibrin during fibrinolysis	24-26,91-94
Acylplasmin	^{99m}Tc	as with plasmin	95
Plasminogen	^{131}I, ^{125}I	conversion to plasmin which then binds to fibrin	44,96
Tissue Plasminogen Activator	^{111}In	activates plasminogen to form plasmin	97
Streptokinase	^{99m}Tc, ^{131}I, ^{125}I	enzyme which converts plasminogen, adsorbed to fibrin, into plasmin	27,28,30,98,99
Urokinase	^{99m}Tc, ^{131}I	fibrinolytic enzyme activating plasminogen	29,30
Anti-fibrinogen Monoclonal Antibody	^{131}I	antibody which binds to fibrinogen	45
Anti-fibrin Monoclonal Antibody	^{131}I	antibody which binds to fibrin	46

Anti-platelet Monoclonal Antibody	^{99m}Tc, ^{111}In, ^{123}I	antibody which binds to platelets	47,100,101
Leukocytes	^{111}In, ^{123}I, ^{51}Cr	invasion into inflamed thrombotic region	102-106
Sulphur Colloid	^{99m}Tc	soft tissue edema; nonselective binding to thrombi; phagocytosis by leukocytes	107-110
^{67}Ga-Citrate		incorporation into intracellular structures of leukocytes	111
Albumin Macroaggregates and Microspheres	^{99m}Tc	combination of factors: adhesion, adsorption, and mechanical entrapment; detect changes in dynamic studies in venous pool due to presence of thrombus	33-35,112
Red Blood Cells	^{99m}Tc	as with albumin macroaggregates	15,31,32,113,114
Albumin	^{99m}Tc	venous blood pool imaging; detect changes in dynamic blood flow	115
Sodium Pertechnetate	^{99m}Tc	as with albumin	116,117

A. Fibrinogen

Fibrinogen has been defined as

> "that protein in blood and tissue extract which in the presence of thrombin is transformed into an insoluble product which is called fibrin" (123).

This protein occurs in blood plasma of most vertebrates at concentrations in the range of two to four grams per litre (124) and, with a molecular weight of 340,000 ± 20,000 daltons (125), is considered to be one of the larger macromolecules in plasma. The generally accepted values of other physicochemical parameters as determined for human fibrinogen are listed in Table 2 (123,124,126,127).

Amino acid analysis of fibrinogen performed by Cartwright and Kekwick (128) revealed that more than 90% of the protein consisted of amino acids. Only 4.5% of the weight is composed of covalently bound carbohydrates which in turn are made up of neutral hexoses, glucosamine, and sialic acid (129). The molecule also contains small amounts of phosphate and sulphate esters (123). Fibrinogen has no free sulphydryl groups, all of its cysteine being involved in disulphide bridges (124).

Following sulphitolysis of the disulphide bridges, Henschen (130) was able to isolate the composite polypeptide chains of fibrinogen. Since the intact molecule was shown to contain six chains, and as only three types of chains could be demonstrated, it appears that the molecule is composed of three polypeptide chains in a paired dimer held together by three symmetrical disulphide bonds (131). Molecular weight values for the chains suggest a minimum molecular weight of 170,000 daltons for a fibrinogen unit. Consequently, the molecular weight of 340,000 daltons attributed to the intact fibrinogen is satisfied by the formula $(A\alpha, B\beta, \gamma)_2$.

The fibrinogen-fibrin transformation takes place after removal of two acidic peptides from the NH_2-terminal portion of the $A\alpha$- and $B\beta$-chains (123). Thrombin, a trypsin-like enzyme, cleaves the arginyl-glycyl bonds thus forming two peptides, namely fibrinopeptides A and B. These peptides then rearrange themselves exposing a structure which is active in polymerization. Fibrinogen is a very labile protein, being denatured at temperatures as low as

Table 2

Physicochemical Parameters of Human Fibrinogen (123,124,126,127)

Molecular weight	340,000 ± 20,000 daltons
Sedimentation coefficient (S_{20},w)	7.9 S
Translational diffusion coefficient (D_{20},w)	$2.0 \cdot 10^{-7}$ cm^2 s^{-1}
Rotary diffusion coefficient (θ_{20},w)	40,000 s^{-1}
Intrinsic viscosity [η]	0.25 dL/g
Partial specific volume ($|\nu|$)	0.71 - 0.72 mL/g
Frictional ratio (f/f_0)	2.34
Molecular volume	$3.7 \cdot 10^3$ nm^3
Electrophoretic mobility (μ)	$2.1 \cdot 10^{-5}$ cm^2 s^{-1} V^{-1}
Extinction Coefficient ($E_{1cm}^{1\%}$, 280 nm)	15 - 16
Isoelectric point (IEP)	5.5
Percent α-helix	33

47°C. In addition, pH extremes, isolation, radiolabeling and storage procedures readily denature this protein (126,132).

B. Radioiodinated Fibrinogen

1. Fibrinogen Uptake Test (FUT)

The concept of using radioiodinated fibrinogen was first introduced in 1957 by Ambrus *et al* (133) who produced radioactive thrombi in animals by injecting ^{131}I-labeled fibrinogen followed by thrombin into occluded vessel segments. Hobbs and Davies (134) also observed that radioiodinated fibrinogen accumulated in areas of venous thrombosis and suggested that this approach could form the basis of a clinical test for the early detection of venous thrombi. Palko *et al* (135) confirmed the results of Hobbs and Davies using ^{131}I-fibrinogen in experimental animals and in man; however, it was Atkins and Hawkins (136) who, in 1965, successfully used ^{125}I-labeled fibrinogen in patients with deep vein thrombosis.

Flanc *et al* (137), and Negus and coworkers (138) refined the method used by Atkins, and employed venographic confirmation to establish the value of FUT for the detection of clinically silent thrombosis in post-operative patients and patients with suspected DVT. They showed that 90% of thrombi found by phlebography could be detected by the FUT test. Kakkar *et al* (38,62) simplified the test so that it could be adapted to screen a large number of patients and be performed at the patient's bedside.

The procedure consists of marking points along the leg from the groin down along the femoral vein in the thigh, and also down the posteriormedial aspect of the calf. Twenty-four hours prior to injection the patient's thyroid gland is blocked by sodium iodide (100 mg) given orally to prevent uptake of the tracer. ^{125}I-fibrinogen (100 μCi) is first injected into an arm vein. Radioactivity is then measured using a hand-held NaI(Tl) scintillation system, the output of which is fed into a scalar and timer, or to a ratemeter. The crystal probe is placed over the heart, the radioactivity measured, and the instrument adjusted to a reading of 100%. The legs are then scanned with this probe over each marking. To determine whether an increase in radioactivity has occurred at any point on the leg, results may be compared either to an adjacent point on the same leg, to a corresponding point on the opposite leg, or to earlier measurements of the point itself. A relative increase of 20% in any value suggests the formation of a thrombus. Radioactivity is measured at daily intervals up to six days after the initial injection (48).

Since the development of the scintillation probe monitors, the ^{125}I-FUT test has been demonstrated to be an excellent epidemiologic tool in patients at risk of venous thrombosis, and to be generally accurate in patients with suspected thrombosis when compared with contrast venography (139,140). The ^{125}I-FUT test has both high sensitivity (94%) and high specificity (93%) for thrombi that form in the distal half of the thigh, the popliteal fossa, or calf (62). Unfortunately, it is unable to detect venous thrombi forming in the pelvis or upper thigh due to accumulation of radioactive iodine in the bladder, and attenuation by the tissue of the weak 27 and 35 keV photon emissions of ^{125}I. This would explain why some investigators found substantial discrepancies between contrast venography and the ^{125}I-fibrinogen uptake test, particularly in those patients where thrombi were predominantly located in the pelvic area (141,142).

Since fibrinogen is only accumulated in biochemically active thrombi, ^{125}I-fibrinogen would be of little use in detecting those thrombi which have stabilized or are being lysed. Thus only rarely can thrombi be identified in patients who have had symptoms for more than one week (17).

Another limitation is that the scintillation probe does not provide images of the spatial distribution of radioactivity; thus, sources of increased uptake cannot be differentiated. This could lead to false positive studies in those patients with ruptured muscle fibres, hematomas, arthritis in the knee, fractures, ulcers, gross edema, surgical sites, and infections in the calf (143), i.e. areas where fibrinogen and fibrin may accumulate.

Two other disadvantages of the fibrinogen uptake test are de19,140):

1. reliable interpretations can be made no sooner than 24 hours post-injection, and the study may last up to one week before a definitve diagnosis can be made, and

2. the possibility of the patient acquiring a hepatitis virus (when non-autologous fibrinogen preparations are employed).

Even with these limitations, when combined with impedance plethysmography ^{125}I-fibrinogen can be as accurate as contrast venography (143).

2. ^{131}I-Fibrinogen

Due to the attenuation of the low energy photons of ^{125}I by overlying tissues (144,145), several investigators have labeled fibrinogen with ^{131}I for its use in the detection of thrombi (64,146). Charkes *et al* (65) investigated the use of scintiscanning with homologous ^{131}I-fibrinogen using a portable rectilinear scanner in 37 patients following surgery. Scans were performed up to 7 days after injection with 350 μCi of ^{131}I-fibrinogen. Compared with phlebography an accuracy of 93%, sensitivity of 70%, and specificity of 95% was obtained. They found that the ability to visualize deep-seated thrombi using a scanner was an advantage over the scintillation probe employed in ^{125}I-FUT. However the prolonged scanning time of 1.5 hours per study and the inability to distinguish between blood pooling in varicosities and transient DVT were limitations of ^{131}I-fibrinogen.

Prescott *et al* (40) also studied ^{131}I-fibrinogen using autologous fibrinogen in 20 patients. When venography was used as the reference diagnostic standard, scanning had a sensitivity of 67%, and a specificity of 95%. They failed to demonstrate the ability of ^{131}I-fibrinogen to detect thrombi in the pelvic veins and speculated that this was likely due to background radioactivity in the bladder. Thus they concluded that there was no advantage in using ^{131}I-fibrinogen over ^{125}I-fibrinogen in detecting deep vein thrombosis.

There are several other disadvantages of ^{131}I-fibrinogen which preclude its utilization as a thrombus imaging agent (39). Beta emission from ^{131}I decay results in a substantial radiation burden to the patient when combined with a protein such as fibrinogen which has a long biological half-life. Therefore the dose administered must be limited which results in a prolonged imaging procedure. Also, multiple energy photons of ^{131}I degrade the spatial resolution. In fibrinogen scintigraphy, this is a critical factor since small lesions such as thrombi, which have a low target to non-target ratio, must be discriminated.

3. ^{123}I-Fibrinogen

DeNardo *et al* (66,147) have demonstrated the potential usefulness of ^{123}I-fibrinogen in association with imaging as a method for detection of deep vein thrombosis. The desirable characteristics of ^{123}I (i.e. a single photon emission of 159 keV, 13 hour half-life, and a low absorbed radiation dose) make it well suited for nuclear medicine imaging.

More than 350 ^{123}I-fibrinogen scintigraphic studies were evaluated by DeNardo *et al* (39). Patients were injected, 6 to 24 hours prior to scintigraphy, with 1.5 to 4.0 mCi (55.5 to 148 MBq) of ^{123}I-fibrinogen containing 1 to 2 mg protein. Anterior scintigraphy was performed with a scintillation camera and total body scanning table. Iodine-123-fibrinogen scintigraphy was found to be more sensitive than ^{125}I-FUT or radiopaque venography. Total body scintigraphy provided the opportunity to assess the entire venous system and imaging could be completed within 30 minutes. Furthermore, the spatial resolution allowed hematomas and other conditions which lead to fibrinogen-fibrin deposition to be be readily differentiated from thrombophlebitis. These investigators were able to detect thrombophlebitis within 4 to 6 hours after injection although the slow blood clearance of ^{123}I-fibrinogen frequently caused 16 to 24 hour scintigraphs to be more readily interpreted. This was considered to be a disadvantage in view of the physical half-life of ^{123}I and the desire for an early diagnosis.

DeNardo and associates (67) also compared ^{123}I-fibrinogen bioscintigraphy with the ^{125}I-FUT and contrast venography and found that bioscintigraphy appeared to be an accurate and effective procedure. The accuracy, sensitivity, and specificity were 90%, 92%, and 87% respectively. Yet these results were not significantly different from either venography or FUT.

^{123}I-fibrinogen bioscintigraphy does offer considerable promise as a noninvasive method for the detection of venous thrombosis. However, protein-iodination grade ^{123}I is costly due to its method of production requiring large cyclotrons (148). Also, in the case of epidemiologic studies, multiple injections of ^{123}I-fibrinogen would be required because of the short physical half-life of ^{123}I thus increasing the radiation dose to the patients (67).

4. Highly Iodinated Fibrinogen

The usual procedure for iodination of fibrinogen involves electrophilic substitution of an average of 0.5 to 1 iodine atoms per molecule of fibrinogen (119). At this level of iodination, labeled fibrinogen retains its biological characteristics and will behave *in vivo* most like authentic fibrinogen. However, due to the long biologic half-life of radioiodinated fibrinogen, high background radioactivity in patients shortly after injection reduces the likelihood of detecting thrombi in areas such as the pelvis.

Thus Harwig *et al* (149-151) prepared highly iodinated fibrinogen, i.e. fibrinogen labeled with both ^{123}I and stable iodine, to an extent of 25 to 100 iodine atoms per molecule. Labeling was performed by electrolysis with the resultant product being indistinguishable from that of unlabeled fibrinogen with respect to its molecular weight profile and clottability. The high levels of iodination resulted in faster clearance of ^{123}I-fibrinogen, both initially and after 24 hours, and thrombus to blood ratios of 50:1 were obtained (approximately twice as high as regularly iodinated preparations). Visualization of an 8 hour old thrombus was achieved with ^{123}I-labeled highly iodinated fibrinogen as early as 4 hours after injection despite high blood background radioactivity. The bladder did not appear to interfere with visualization of the thrombus.

In 1976, Colombetti (152) reported the preparation of high specific activity ^{123}I-fibrinogen using the electrolytic method developed by Harwig *et al* (149). When injected into dogs, thrombi were clearly shown in the legs. Pelvic thrombi were also delineated 4 hours after injection although the high blood background generally led to insufficient differentiation between the thrombus and soft tissue.

5. Disadvantages of Radioiodination of Fibrinogen

Several agents have been employed for the radioiodination of fibrinogen. These include Chloramine-T, lactoperoxidase, electrolysis, iodine monochloride, and Iodogen™ (127,153-155). All involve oxidation of radioiodide to an electropositive state; electrophilic aromatic substitution then occurs introducing the iodine atom mainly into tyrosine residues, although histidine and tryptophan residues may also become labeled (156). Each of these radiolabeling methods has its advantages and disadvantages, these being related to the complexity of the labeling procedure, yield, specific activity, radiochemical stability and degree of protein denaturation during radiolabeling.

Although the incorporation of iodine into proteins can be easily achieved, radioiodination has several drawbacks (156):

1. the presence of oxidizing and reducing agents can cause significant damage to the protein and cause a loss of its biologic activity,
2. radioiodinated proteins are subject to radiation damage causing gradual decomposition or denaturation of the biologic molecule, thus resulting in a limited shelf-life of the compound, and
3. due to the biologic hazard of working with radioiodine, a suitably equipped laboratory is absolutely essential. Proper handling techniques, personnel monitoring, and shielding are necessary.

Due to the limitations described above, various other radionuclides were investigated for the radiolabeling of fibrinogen.

C. Radiolabeling Fibrinogen with Radionuclides other than Iodine

1. ^{77}Br-Fibrinogen

As a tracer, ^{77}Br has several advantages over iodine. For instance, bromine forms a stronger bond with carbon than does iodine (157). Also, while free radioiodide, released by hydrolysis from a compound, concentrates in the thyroid, free bromide is not concentrated by any organ (158). Bromine-77 decays 99% by electron capture (159) with major gamma emissions at 242 keV (30%), 300 keV (6%), 520 keV (24%), and 580 keV (7%). The 57 hour half-life of ^{77}Br makes it suitable for certain nuclear medicine procedures such as fibrinogen turnover studies (160). When used as a plasma tracer the radiation dose from ^{77}Br would only be about one-tenth the dose delivered by either ^{125}I or ^{131}I (161).

Thus, in order to provide a chemically more stable alternative to radioiodinated fibrinogen and to decrease the radiation dose associated with the use of ^{125}I and ^{131}I, Knight *et al* (68) labeled fibrinogen with ^{77}Br by an indirect halogenation technique. The two-step radiolabeling procedure, similar to the Bolton-Hunter iodination method (162), involved bromination of an acylating agent, N-succinimidyl-3-(4-hydroxyphenyl)propionate (SHPP) followed by conjugation of the purified ^{77}Br-SHPP with fibrinogen under mild conditions. With this method, conjugation yields ranged from 35% to 50%, with higher yields when more concentrated fibrinogen solutions were employed. An isotopic clottability of greater than 90% was attained and the compound was completely stable with respect to *in vitro* dehalogenation for over seven days at room temperature in neutral buffer. Unfortunately, indirect bromination of fibrinogen was more difficult than when a direct method was employed.

Therefore, McElvany *et al* (69) introduced a new brominating enzyme, bromoperoxidase, which appeared to catalyze bromination at a pH optimum near neutrality. *In vitro* clottability of ^{77}Br-fibrinogen was reported to be greater than 95%, these results being consistent with those reported by other workers for the radioiodination of fibrinogen (153,155). ^{77}Br-fibrinogen cleared from plasma at a much slower rate compared to ^{125}I-fibrinogen, with a biologic half-life approximately 3.5 times that of iodinated fibrinogen. Although *in vitro* stability studies indicated that the product was stable, the radiolabeling efficiencies obtained under optimum conditions remained low, ranging between 50 to 60%.

2. ^{197}Hg-Labeled-Fibrinogen

Radiolabeling of fibrinogen with mercury-197 has also been attempted by Saha and coworkers (70). Optimum yields of 90 to 95 percent were obtained by incubating 10 mg of fibrinogen at room temperature with 2 μCi of $^{197}HgCl_2$ for 30 minutes at physiologic pH. Yields increased with an increase in pH, temperature, and protein concentration. However, the radiolabeling efficiency dropped dramatically when attempting to increase the specific activity of ^{197}Hg-fibrinogen which, the authors speculated, was due to an increase of non-radioactive mercury atoms present in $^{197}HgCl_2$. Assessment of clottability also revealed that only 50 to 60 percent of the radiolabeled protein remained biologically intact.

3. ^{111}In-Oxine-Fibrinogen

Ramberg-Laskaris *et al* (71) reported that fibrinogen could be successfully labeled with ^{111}In-oxine. Labeling efficiencies ranged from 28.8% at a pH of 5, to 55.5% at pH 10. Increasing the temperature from 4°C to 37°C caused a decrease in the protein's clottability from 47.7% to 32.1%. The percent clottability was found to increase with increased ^{111}In-oxine concentration: 28.9% at 0.8 μCi/mL to 50.2% at 25 μCi/mL. *In vitro* stability studies were not reported; however, biodistribution of ^{111}In-oxine-fibrinogen in baboons, using a gamma camera, showed circulating activity with uptake by both the liver and spleen. No thrombus localization studies were performed to demonstrate the effectiveness of this radiopharmaceutical in localizing in active thrombi.

4. ^{99m}Tc-Fibrinogen

Technetium-99m would be a very desirable label for fibrinogen thrombus scintigraphy due to its availability, its abundant photon emission of 140 keV, half-life of 6 hours, and absence of primary beta emission (163,164). These potential advantages have led several laboratories to attempt labeling fibrinogen with ^{99m}Tc.

In 1975, Wong and Mishkin (165) reported that exogenous fibrinogen had been successfully labeled with ^{99m}Tc using a modified electrolytic method using zirconium electrodes in an acidic medium. Although an average binding efficiency of 76.4% was achieved, the protein was only 25% clottable. This, the authors proposed, was due to manipulation of the protein during purification of the product by ammonium sulfate precipitation, or extraction with glycine. In addition, the low pH of 2 to 4 employed, as well as the presence of ^{99m}Tc-zirconium species formed after hydrolysis may have contributed to the denaturation process. Denaturation also occurred during pH adjustment with buffering agents.

In an attempt to improve the radiolabeling efficiency using electrolysis, Harwig and researchers (74) prepared ^{99m}Tc-fibrinogen at a pH of 6. The method employed tin electrodes with *in situ* production of the stannous ion as an intermediate reducing agent for $^{99m}TcO_4^-$. The final ^{99m}Tc-fibrinogen radiolabeling efficiency was 70 to 80% with an isotopic clottability of 50 to 65%. The *in vivo* behaviour of this product was, however, more disappointing with only 25% of the injected dose remaining in the circulation after 10 minutes (166). The nonclottable labeled protein cleared more rapidly than the biologically intact radiolabeled protein. Hence, the fraction of clottable material increased with time, and the thrombus:blood ratios were higher than those for radioiodinated fibrinogen under the conditions tested.

Jeghers *et al* (50) proposed a chemical method of labeling fibrinogen. Briefly, ^{99m}Tc was reduced by stannous chloride and the labeling was allowed to take place in an alkaline medium, the optimal pH being 10.4 to 10.5. After an incubation period of one hour, the pH was brought down to neutrality by the addition of citrate buffer. When correctly executed the radiolabeling yield achieved was 92 to 95%, and both the *in vitro* and *in vivo* clottability was approximately 72%. Twenty-four hours post-injection, approximately 28% of the radioactivity was recovered in the clot. Ten percent of the radioactivity in the radiolabeled product was not protein bound and contributed to the poor quality of the scintigraphic images in human subjects.

Other investigators (167,168) have also used a similar chemical method for labeling fibrinogen with ^{99m}Tc. The radiolabeling yield and clottability values generally agreed with those obtained by Jeghers and coworkers (50). Yet due to the high blood background, scintigraphic images were only of value 24 hours after injection. In addition, increased radioactivity accumulating at venous junctions, varices in the calf, and, in general, any enhanced venous network caused diagnostic problems leading to false positive results. Jonckheer *et al* (169) speculated that since only a small portion of the thrombus is in contact with circulating blood and actively involved in the dynamic process of fibrin formation, false negative results could also occur. Spicher and associates (51) demonstrated that rapid dissociation of the radiolabel from the protein took place in plasma. Thus this could also contribute to poor quality scintigraphic images.

Finally, Sandler *et al* (18) found that ^{99m}Tc-fibrinogen scans were 80% accurate when compared with contrast venography and only 68% of the scans could be reported as unequivocal with respect to thrombus detection. Therefore, they suggested that ^{99m}Tc-fibrinogen was not a suitable method for the definitive diagnosis of deep vein thrombosis.

D. Bifunctional Chelating Agents

All the radiolabeled fibrinogen preparations discussed to this point were labeled in one of two manners: by covalent substitution with elements such as iodine or bromine, or by chelation with radiometals. However, direct labeling of a protein such as fibrinogen with a metallic radionuclide suffers from several weaknesses (170,171):

1. the radionuclide may bind to the protein with insufficient affinity to produce a stable bond,
2. the radiolabel may bind to the native functional groups of the protein needed to retain its biological activity. Should the radionuclide interfere with this, the normal behaviour of the molecule would be altered, and
3. the specific activity attained may not be high enough to achieve a high target to non-target ratio. For example, high thrombus to blood ratios are necessary to be able to detect thrombi in areas of high venous flow such as in the pelvic areas.

An alternate approach may be to link metal-chelating groups to the protein which may then bind the desired radiometal. A method such as this would add flexibility in the chemical reactions involved, the choice of radioisotope, and the time of addition of the radionuclide (172).

The first approach to covalently couple chelating groups to macromolecules was reported by Benisek and Richards (173) who reacted lysozyme with methyl picolinimidate to produce a bidentate chelating site. In 1974, Sundberg *et al* (52,174) utilized a bifunctional analogue of ethylenediaminetetraacetic acid (EDTA) to create specific metal chelating sites on biological molecules. The synthesis was later elaborated by Yeh and coworkers (175) using α-amino acids as the starting materials. The metal chelators are most often derived from polyaminocarboxylic acids such as EDTA, or diethylenetriaminepentaacetic acid (DTPA) (55) because of their large formation constants with a large variety of metal ions and their relative ease for synthetic manipulation.

In general, bifunctional chelating agents all contain two types of functional groups. One group is always a multidentate metal chelating ligand. The other functional group can be of various types (176):

1. a reactive moiety capable of forming covalent bonds with biological molecules,
2. a hydrophobic aliphatic chain which is likely to incorporate itself into a biological membrane,
3. a haptenic molecule with affinity for an antibody, or
4. a substrate or inhibitor molecule preferentially bound to a specific enzyme.

Thus the role of the second functional group is to direct the chelating agent to a region of interest in a biological system.

1. Advantages of Chelate-Tagged Molecules For Imaging

One of the main advantages for the development of this form of radiolabeling is the availability of isotopes with convenient half-lives and useful radiation for imaging, such as ^{111}In, ^{99m}Tc, and ^{67}Ga. Another reason for using this technique is that the synthetic chemistry is separate from the radiochemistry (172). Proteins may be chemically modified, purified, and stored in nonradioactive form. The radioactive metal ion may then be added. In this way a high specific activity may possibly be achieved, since radiolabeling can be performed immediately before utilization of the radiopharmaceutical. In addition, the radiation absorbed dose to personnel may be reduced when employing this method, as opposed to the radioiodination of protein.

Protein-metal conjugates prepared using bifunctional chelating agents are quite stable (177). Chelated metal ions from metabolized proteins are often rapidly excreted by the kidneys (172) thus, in theory, reducing the radiation dose to the patient. When labeled properly, the proteins may have biological half-lives comparable to the radioiodinated proteins or their native counterparts (178).

An attractive feature of bifunctional chelating agents is the versatility of the chemistry involved in the modification of proteins (175,176). With an increased choice in the type of chemical reaction employed, it is possible to formulate a compound with high specific activity and minimum loss of the macromolecule's biologic activity.

2. Desirable Properties of Bifunctional Chelating Agents and Metal Ions

Two general requirements are essential for bifunctional chelating agents to be effective in biological applications (176):

1. the chelating agent must be attached to the protein in such a way that the product retains the original biological properties of the protein, and also, the original metal-binding properities of the chelating agent, and
2. the metal ion must bind with the chelating agent such that dissociation will occur very slowly or not at all under *in vivo* and *in vitro* conditions.

The serum protein transferrin is well known for its rapid removal of some coordinated metal ions from chelates *in vivo* (179,180). Radioactive metals bound to transferrin subsequently localize in the liver and bone marrow producing undesirable background activity. Consequently, if radiopharmaceuticals prepared with bifunctional chelating agents are to be used for clinical studies, the metal chelate must prove to be kinetically inert and stable *in vivo* (172).

E. Types of Bifunctional Chelating Agents

1. Diethylenetriaminepentaacetic Acid (DTPA) Derivatives

Original work with DTPA derivatives involved formation of the mixed carboxycarbonic anhydride by reaction with triethylamine and isobutylchloroformate (55). This chelating agent was then added to human serum albumin (HSA), which was purified by exhaustive dialysis and gel chromatography to remove excess DTPA, and subsequently labeled with ^{113m}In. The resultant radiolabeling yield was found to be greater than 98% and the product behaved similarly to ^{125}I-HSA in mice. The drawback to this procedure is that six individual steps are required to prepare the final radiolabeled protein complex. Also, hydrolysis of the anhydride prior to protein conjugation could significantly reduce the radiolabeling efficiency. This procedure has been reported to be successful in radiolabeling monoclonal antibodies for myocardial infarct imaging (181), radiolabeling albumin microspheres for lung imaging (54), and for tumour imaging (182).

Khaw and coworkers (75) have described a three-step process for the preparation of ^{99m}Tc-DTPA-fibrinogen. Fibrinogen was first modified using DTPA anhydride prepared by the above method (55). The technetium was reduced with sodium dithionate and then added to the buffered protein. *In vitro* coagulability of the final product approximated 86% and biodistribution studies showed that 67% of the radioactivity remained in the blood after 15 minutes, this value decreasing to 11.5% after 24 hours. Less than one percent of the dose localized in the thyroid indicating that the product was stable *in vivo* over the 24 hour period studied.

Although this particular radiopharmaceutical was reported to have a higher coagulability and a slower whole blood clearance than the ^{99m}Tc-fibrinogen prepared by Harwig (166), the preparation of ^{99m}Tc-fibrinogen was rather labour intensive, requiring four purification steps after radiolabeling to remove contaminating colloids, thus reducing the percent yield to 31.9%. Also, Khaw (75) reported that excessive amounts of DTPA anhydride caused varying degrees of denaturation of the derived fibrinogen.

Hnatowich *et al* (183) have prepared bicyclic DTPA anhydride by heating DTPA and excess acetic anhydride in pyridine. The anhydride was added to protein as a solid or in a chloroform solution. Using this method, Hnatowich and associates (184,185) have labeled albumin microspheres and a monoclonal antibody to carcinoembryonic antigen successfully with ^{67}Ga and ^{111}In respectively.

Layne *et al* (42) have also prepared ^{111}In-DTPA-fibrinogen utilizing the cyclic anhydride of DTPA and found the clotting time of the radiolabeled fibrinogen to be identical to that of native fibrinogen regardless of whether an anhydride to protein ratio of 1:1 or 5:1 was employed. Blood clearance studies and thrombus to blood ratios of radioactivity using ^{111}In-DTPA-fibrinogen in canine models yielded similar data as that obtained with ^{125}I-fibrinogen. Unfortunately, the labeling of fibrinogen with ^{111}In, by first modifying the protein with derivatized DTPA dissolved in chloroform, led to a low radiolabeling efficiency of 13%.

This prompted Lavie *et al* (72) to modify Layne's procedure, by dissolving DTPA anhydride in dimethyl sulfoxide and then attaching it to the protein. The modified protein was labeled with ^{111}In-acetate producing an average yield of 90% at a molar ratio of 0.1:1 DTPA:fibrinogen. At a reagent-protein ratio greater than 1:1, the radiolabeling efficiency dropped to 70%. The final product obtained was highly clottable and stable *in vitro* (72).

^{67}Ga-DTPA-fibrinogen has been prepared by Saha and Halbleib (76) and its biodistribution studied in Balb/c mice. Again, DTPA anhydride powder was employed by mixing the chelating agent with lyophilized fibrinogen at a molar ratio of 5:1, and then dissolving the mixture in 0.05M Hepes buffer at pH 7. The DTPA-fibrinogen complex was then purified by column chromatography, labeled with ^{67}Ga, and further purified by gel filtration. The radiolabeling efficiency obtained ranged from 60 to 85%, and the clottability remained constant at 61% for 24 hours. Only 6% of the radioactivity was found in the blood 16 hours after injection, with the majority of the radioactivity localizing in the kidneys and liver.

Recently, the use of a succinimide ester of DTPA has been introduced as an alternative to the anhydride (186,187). However as yet, this derivatized chelating agent has not been investigated as a method for labeling fibrinogen.

2. Ethylenediaminetetraacetic Acid (EDTA) Derivatives

In the original work by Sundberg and associates (52) an EDTA derivative was chosen over DTPA because of its less complicated synthesis in spite of the fact that DTPA possessed higher binding constants for metals. These researchers synthesized the intermediate compound 1-(*p*-aminophenyl)-EDTA, which could subsequently be acylated, alkylated, or otherwise modified to form either biologically active derivatives or covalent labeling reagents (52). For instance, the above intermediate compound could be derivatized to produce a bromacetamido group, an isothiocyanate group, or a diazonium salt (172).

Diazonium salts couple readily with lysyl, tyrosyl, and histidyl residues (188), the predominant amino acid modified being dependant upon pH (189). Thus non-selective binding of the diazotized conjugate with proteins will occur. Isothiocyanate-EDTA derivatives also label proteins randomly producing higher yields under milder conditions as compared to the diazonium complexes (172). In contrast, bromacetamido-benzyl-EDTA specifically labels sulphydryl groups (178). Thus if a protein contains a single sulphydryl group that is not essential for activity a theoretical yield of one chelate per protein molecule can be achieved. All of these complexes have been found to form stable conjugates with proteins *in vitro* (172).

Fibrinogen has also been labeled with EDTA derivatives. Sundberg and associates (52) conjugated fibrinogen with 1-(*p*-benzenediazonium)EDTA and then labeled the complex with ^{111}In. Their *in vitro* studies of indium binding by perturbed angular correlation demonstrated that indium bound quantitatively and rapidly to fibrinogen. They did not, however, report the evaluation of this compound as a potential thrombus imaging agent.

Goodwin *et al* (73) also reported a method for labeling fibrinogen with ^{111}In via the azophenyl-EDTA complex. Radioindium was first complexed with the EDTA portion of the molecule and then linked to the protein by means of the diazo group. With a conjugation level of three equivalents ^{111}In-azophenyl-EDTA per mole fibrinogen, the resultant complex obtained an *in vitro* clottability of 80%. Again, no thrombus images were reported using this radiopharmaceutical.

F. Deferoxamine

Although EDTA and DTPA derivatives produce stable protein conjugates, they are, in general, characterized by complicated syntheses and purification procedures. In the case of conjugated fibrinogen, the labeling yield is often quite low. Therefore, another type of chelating agent, deferoxamine, has been extensively investigated for the preparation of radiometal labeled macromolecules.

Deferoxamine B is a naturally occuring iron-chelating hydroxamic acid which is synthesized and secreted by many prokaryotes (57). This sideramine possesses growth-promoting properties (58) and appears to exert an important function in the iron metabolism of microorganisms due to its ability to solubilize extracellular iron metal and iron oxides (190), with subsequent incorporation of iron into the porphyrin systems (58). Deferoxamine B is produced when the complex-bound trivalent iron contained in ferrioxamine is removed by chemical means.

1. Physicochemical Properties of Deferoxamine

Deferoxamine is soluble in water and alcohol, and practically insoluble in organic solvents such as chloroform and ether (191). It strongly absorbs ultraviolet light at approximately 220 nm, and when complexed with iron, absorption at 430 nm ($E^{1\%}_{1cm}=39$) is observed due to the formation of a red-brown colour (192). Deferoxamine contains one amino group which accounts for the basic character of the compound, and enables it to form salts with organic and inorganic acids (58). The molecular weight of deferoxamine base has been calculated to be 560.7 daltons.

2. Chemistry

Deferoxamine is composed of one unit of acetic acid, two units of succinic acid and three units of 1-amino-5-hydroxylaminopentane (Figure 1) (58). The three organic groups are interlinked to form a chain in which there are three hydroxamic acid moieties inside, and the free amino group at the end. Reaction of the desferricompound with a ferric ion involves the displacement of three protons from the hydroxamic acid groups. These groups then rearrange themselves in such a way that their six oxygen atoms form the apices of an octahedron with the iron atom at the centre (Figure 2) (58,193). In this state, the molecule has no net charge and all six coordination valencies of the metal ion are satisfied (193). This shell of organic material thus results in an iron complex of very great stability. The stability

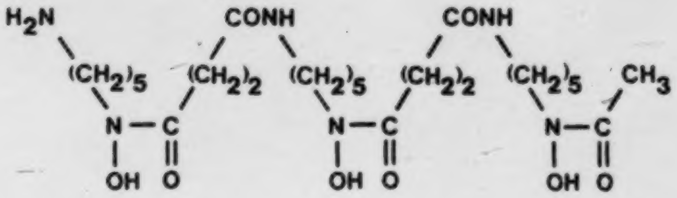


Figure 1: Structure of Deferoxamine (58)

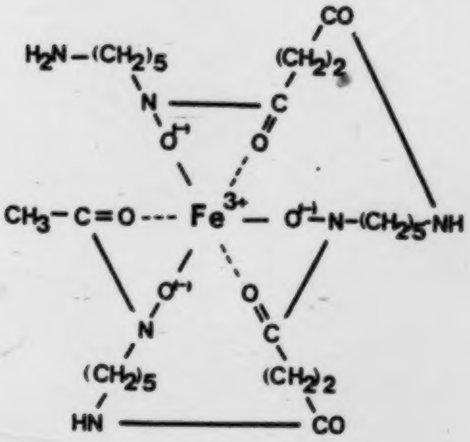


Figure 2: Structure of Ferrioxamine (58)

constants for various deferoxamine-metal complexes compared with other chelating agents are listed in Table 3 (59,60,194,195). As is illustrated in the table, ferric-deferoxamine is one of the more stable complexes with a formation constant of log K=30.6. In contrast, deferoxamine shows significantly less affinity for ferrous ions.

Keberle (58) reported that deferoxamine was able to extract iron from proteins such as ferritin and hemosiderin *in vitro* to its maximum theoretical binding capacity. However, only 10 to 15 percent of iron was removed from totally saturated transferrin. Yet there was no demonstrable exchange of iron from ferrioxamine to transferrin. *In vivo* studies in dogs

Table 3

Stability Constants (log K) (59,60,194,195)

Metal Ion	Deferoxamine	EDTA	DTPA	Citrate	Transferrin
Fe^{3+}	30.6	25.1	28.0	11.4	30.3
Ga^{3+}	>30.6	20.3	25.5	10.0	23.7
In^{3+}	<30.6	25.0	29.0	6.2	30.5
Al^{3+}	21.5	16.3	18.6	3.5	
Cu^{2+}	14.1	18.8	21.6	5.9	
Pb^{2+}	10.7	18.0	18.8	4.4	
Co^{2+}	10.3	16.3	19.3	5.0	
Zn^{2+}	10.1	16.5	18.4	4.9	
Fe^{2+}	7.2	14.3	16.5	4.4	
Ni^{2+}	10.9	18.6	20.3	5.4	
Cd^{2+}	7.9	16.5	19.2	3.8	
Mg^{2+}	4.3	8.8	9.3	3.4	
Ca^{2+}	2.6	10.7	10.8	3.5	
Sr^{2+}	2.2	8.7	9.8	3.0	

indicated that ferrioxamine was rapidly excreted by the kidneys via glomerular filtration and partial tubular secretion with 94% of the dose removed after 5 hours. Deferoxamine, however, is metabolized and then excreted mainly in the bile with a metabolic half-life of one hour (58).

3. Uses of Deferoxamine

Due to its great-affinity for ferric ions and its subsequent rapid elimination by the kidneys, deferoxamine has been utilized in those conditions associated with excessive iron storage in the tissues such as haemochromatosis (196) and haemosiderosis (191), and in iron overload following repeated transfusions as in thalassaemia (197). It has also been used for the treatment of acute iron poisoning (198).

4. Potential Use in Radiolabeling of Proteins

Interest in deferoxamine as a potential radiometal chelator stems from the fact that it not only facilitates uptake of ferric ions but also the Group III elements such Al^{3+}, Ga^{3+}, and In^{3+}. The data by Emery (56) support the concept that ^{67}Ga behaves physiologically, in part, as a non-reducible iron analogue. Preliminary investigations by Goodman *et al* (60) demonstrated the relative stability of deferoxamine (DF) complexes: GaDF>FeDF>InDF>CuDF at pH 7.1 and 22°C. Weiner and associates (57) also showed that deferoxamine was more competitive than serum transferrin in the binding of radiogallium, but not radioindium.

Deferoxamine also fulfills the characteristics needed for a bifunctional chelating agent in several ways (199):

1. deferoxamine contains a free amino group of comparatively high reactivity, which may act as a functional group for coupling reactions with proteins,
2. when complexed to protein it forms an uncharged chelate of compact structure; hence its influence on the parent molecule is minimized,
3. compared to EDTA and DTPA derivatives, deferoxamine exhibits a low chelate

stability with cations such as Ca^{2+} and Mg^{2+}, which are essential for many biological reactions,

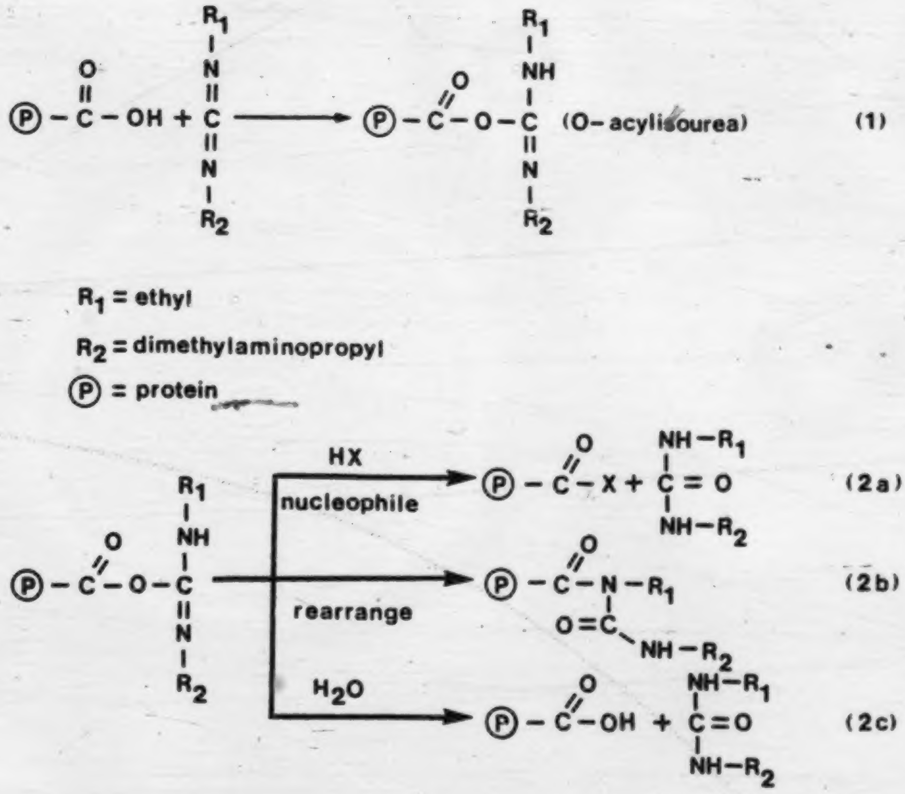
4. it possesses a higher affinity for gallium than iron-binding macromolecules such as lactoferrin and transferrin, and
5. it also satisfies the general requirements as a diagnostic agent due to its low toxicity and easy procurement.

G. Conjugation of Deferoxamine with Proteins

Several methods have been employed to couple prosthetic groups to macromolecules (200-202). Selection of the proper coupling reagent must be determined in order to produce the desired product. In addition, the choice of reagent will depend on the stability range of both the protein and reagent. The reaction conditions need to be extensively studied for each protein, and all the parameters such as temperature, pH, reaction time, and concentration, elucidated to optimize the yield of the modified protein with minimal loss of its biologic activity (203).

1. Water-Soluble Carbodiimides

Water-soluble carbodiimides have become common reagents for the attachment of prosthetic groups to protein carboxyl groups because of the relatively mild conditions used (204). According to Carraway and Koshland (205), the mechanism of the carbodiimide reaction with carboxylic acid groups proceeds as shown in Figure 3. Khorana (206) has postulated that the reaction sequence is initiated by the addition of a carboxyl across one of the double bonds of the diimide system to give an O-acylisourea (equation 1, Figure 3). The activated carboxyl group may then react in several ways. If a nucleophile is present, an acyl-nucleophile product will be produced, plus urea derived from the carbodimide (equation 2a, Figure 3). The O-acylisourea could rearrange itself to form an N-acylurea via an intramolecular acyl transfer (equation 2b, Figure 3). Where the nucleophile is water, hydrolysis occurs with subsequent regeneration of the carboxyl group (equation 2c, Figure 3).



R_1 = ethyl

R_2 = dimethylaminopropyl

Ⓟ = protein

Figure 3: Mechanism of Carbodiimide Reaction with Carboxylic Acids (205)

shown that if the concentration of both the carbodiimide and nucleophile is sufficiently high, rearrangement of the O-acylisourea will be minimized, and the coupling reaction between the protein carboxyl groups and nucleophile will be driven to completion.

The carbodiimide promoted amide formation between protein and chelating agent offers the advantages of a mild, quick, and simple reaction (204). Although any water-soluble carbodiimide may be employed, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) is commonly used, since it is commercially available and relatively stable for long periods of time (204).

Janoki and associates (171) have *utilized* EDC in the preparation of deferoxamine-human serum albumin (DF-HSA). They investigated the effect of selected combinations of molar ratios of the reagents, pH and reaction time on the yield of DF-HSA, and found that use of more than a 100-fold excess of EDC led to a significant loss of protein. Similar results were observed when a reaction time of greater than 60 minutes or a pH above 7 was employed. They rationalized these results by postulating that too great an excess of carbodiimide would activate carbonyl moieties which would favour inter- and intramolecular coupling with nucleophilic groups on the protein itself. According to Riehms and Scheraga (207) some of the crosslinking between a protein's carboxyl and lysine moieties can be reduced by performing the reaction under acidic conditions (pH 4.75). Unfortunately, certain proteins such as fibrinogen are too labile to react under such an acidic climate.

Another limitation of utilizing carbodiimides is that these reagents are not site-specific, thus activating a number of organic functional groups. In aqueous solutions at acidic pH values, the predominant groups on a protein reacting with carbodiimides are carboxyls, sulphydryls (208), and tyrosines (209). The rates of reaction of model sulphydryl and carboxyl compounds with EDC are approximately equal, while tyrosine acts more slowly (205).

Recently, Motta-Hennessy *et al* (210) reported that DF-IgG conjugates prepared via the carbodiimide method of Janoki and coworkers (171) were unstable after storage for more than one week, thus leading to a low radiolabeling yield with ^{67}Ga. In addition, an identity reaction on immunoelectrophoresis was not observed.

2. Glutaraldehyde

In contrast to water-soluble carbodiimides, glutaraldehyde is a bifunctional coupling reagent which may be utilized at neutral pH (211) to couple protein amino groups with an amino moiety of a prosthetic group:

$$\text{protein} - N = CHCH_2CH_2CH_2CH = N - \text{prosthetic group.}$$

Glutaraldehyde is well known for its ability to react with proteins, and the literature on the reaction of glutaraldehyde with biological systems has been extensive. Hopwood (212,213) has reviewed its application in fixation. Several authors have described its use for the preparation of protein-enzyme conjugates (214) and for coupling proteins to various matrices (215,216). Other soluble protein derivatives have also been described (217,218). Glutaraldehyde has been utilized for coupling in two ways (203):

1. a procedure in which the prosthetic group, protein, and glutaraldehyde are reacted in a single step. Conjugates prepared by this method are generally of high molecular weight and are assumed to be heterogenous (i.e. both aggregates and monomeric proteins are formed), and
2. a two-step procedure in which the prosthetic moiety and glutaraldehyde are first reacted, after which the protein is added.

The rationale behind the two-step procedure is that those prosthetic groups containing a low number of free amino moieties are able to bind with glutaraldehyde with minimum crosslinking (219). The free aldehyde group of the glutaraldehyde should then be available for combination with the amino groups of a protein added subsequently, providing that the groups approach closely enough. Using this method, Avrameas and Ternynck (219) successfully coupled horse-radish peroxidase ~~to sheep anti~~-rabbit IgG antibody with little loss of biologic activity.

An excess of glutaraldehyde-conjugate is necessary to allow reaction with the protein to be driven to completion (172). High protein concentrations also enable the glutaraldehyde conjugates to react with protein molecules due to their close vicinity (172). According to Payne (220), high glutaraldehyde and protein concentrations produce extensive derivative formation but not necessarily extensive intermolecular crosslinking. In contrast, low glutaraldehyde concentrations together with large amounts of protein facilitates intermolecular reaction (220). As with all coupling techniques, the exact conditions necessary to obtain the desired extent of conjugation must be determined experimentally for each specific case (172).

As with carbodiimides, glutaraldehyde is not considered a site-specific coupling agent. Although the reactivity of certain groups varies depending on the protein studied, Habeeb and Hiramoto (221) found that when reacting proteins with glutaraldehyde, extensive reaction with N-terminal amino groups occurred. The sulphydryl group of cysteine was also very reactive, whereas the imidazole and tyrosine groups were only slightly reactive. These results were confirmed by Payne (220). Therefore, in proteins which contain no free sulphydryl groups, coupling of glutaraldehyde with protein will occur primarily with the ϵ-amino group of lysine.

The covalent binding of deferoxamine to HSA was reported by Yokoyama *et al* (199) to be easily achieved under mild conditions using the glutaraldehyde two-step method. Using an initial 12 molar excess of deferoxamine-glutaraldehyde conjugate, a final deferoxamine-protein conjugation level of 0.8 moles deferoxamine per mole HSA was obtained. At this molar ratio a 99.8% radiolabeling yield was produced when 0.1 to 5.0 mCi (3.7 to 185 MBq) of ^{67}Ga was added. *In vivo* stability studies in rats showed that ^{67}Ga-deferoxamine-HSA behaved similarly to ^{131}I-HSA. They also found that when ^{67}Ga-deferoxamine-HSA was injected in humans, neither free ^{67}Ga nor ^{67}Ga bound to transferrin was observed over the period of the study.

Janoki and associates (222) also labeled DF-HSA with ^{67}Ga and found that the purified conjugate was able to bind more than 95% of the added ^{67}Ga, 90% of which was still retained after one week of storage. The blood clearance of ^{67}Ga-DF-HSA in rabbits was much slower than that observed with ^{67}Ga-citrate, ^{67}Ga-deferoxamine, and ^{99m}Tc-HSA, but faster than ^{125}I-HSA, with a high amount of radioactivity observed in the liver.

Pritchard *et al* (223) have utilized deferoxamine-glutaraldehyde in the labeling of IgG with ^{111}In. *In vitro* studies showed that the IgG molecule was still able to participate in the antigen-antibody reaction indicating that IgG retained its biologic activity. However, they did find that transferrin was able to remove ^{111}In from deferoxamine when these compounds were incubated in whole serum, suggesting that another radionuclide with stronger affinity for deferoxamine over transferrin be used.

Motta-Hennessy *et al* (210) also employed the glutaraldehyde two-step method to link deferoxamine with human IgG and its Fab fragments. With a conjugation level of 0.9 moles deferoxamine per IgG molecule, achieved by using a 40-fold molar excess of deferoxamine-glutaraldehyde, the modified protein was found to be highly stable *in vitro* and *in vivo* when labeled with ^{67}Ga. The radiogallium remained firmly associated with the protein and there was no significant interaction with blood constituents. In addition, biodistribution studies in mice showed that ^{67}Ga-deferoxamine-IgG closely resembled ^{131}I-HSA with respect to the blood half-life and organ distribution, suggesting that conjugation and radiolabeling did not alter the physiologic properties of the protein. Conjugation also did not interfere with the antigen-antibody reaction, determined by immunoelectrophoresis with antihuman IgG, and by the cell binding assay.

Ohmomo *et al* (77) investigated the possibility of labeling fibrinogen with ^{67}Ga via deferoxamine-glutaraldehyde. Beginning with a 50-fold excess of deferoxamine, a final deferoxamine-fibrinogen conjugation level of three to six moles of deferoxamine per mole of fibrinogen was obtained. One mCi (37 MBq) of ^{67}GaCl$_3$ was added to 2.0 mL of the deferoxamine-fibrinogen solution (2.0 mg/mL protein), incubated for 15 minutes, and then sterilized by passing the radiolabeled protein solution through a 0.22 μm millipore filter. Using this method, a labeling efficiency of 90.3% and isotopic clottability of 85.3% was obtained. The detachment of ^{67}Ga or liberation of ^{67}Ga-deferoxamine from the ^{67}Ga-deferoxamine-fibrinogen molecule was not detected over the period of the biodistribution study in mice. The rabbit blood clearance studies of ^{67}Ga-deferoxamine-fibrinogen showed a slightly higher blood level for a longer period of time as compared to ^{131}I-fibrinogen. Also, venous thrombus scintigraphy in rabbits indicated that both ^{67}Ga-deferoxamine-fibrinogen and ^{131}I-fibrinogen were taken up by the thrombus to a similar extent and could be visualized 3 and 17 hours post-injection. This same group of researchers have also successfully labeled fibrinogen fragment E(1,2) with ^{67}Ga after conjugation with deferoxamine (89). The thrombus to blood ratios achieved in rabbits were 6.3 and 9.1, at 6 and 20 hours respectively, post-injection.

Recently, several research groups in Japan have been developing a novel approach to protein labeling, employing water-soluble polymers containing deferoxamine. By introducing 'spacer' molecules such as dialdehyde starch (DAS) or polyacrolein (PA), which contain many aldehyde groups, a large number of deferoxamine molecules may be linked to labile proteins without loss of the protein's physiologic activity. Using this technique fibrinogen-DAS-deferoxamine was prepared and labeled with ^{67}Ga (78). A specific activity of 0.4 mCi/mg (14.8 MBq/mg) was achieved, and a thrombus to blood ratio of 8.6 was observed in rabbits 24 hours after administration. Both fresh and one day old arterial and venous thrombi could be detected by ^{67}Ga-fibrinogen-DAS-deferoxamine 6 hours after injection (224). In contrast, scintigraphic imaging of thrombi using ^{111}In-oxine-platelets showed accumulation in arterial and venous thrombi 24 hours post-injection. However when employing this radiopharmaceutical in humans, the best scintigraphic imaging of new thrombus formation was obtained 48 to 72 hours after radiolabeled fibrinogen administration (225) due to intense accumulation of radiolabeled fibrinogen in the liver and kidney (226).

III. MATERIALS AND METHODS

A. Preliminary Work

1. Preparation of Heavy-Metal-Free Buffers and Solutions

When utilizing bifunctional chelating agents in radiolabeling procedures, the purification of buffers and ionic reagents is essential since heavy metal contamination will influence the labeling efficiency of the protein-chelate complex (227). The procedure for preparing heavy-metal-free buffers involves the use of Chelex-100™ (Bio-Rad Lab. Ltd, Mississauga, Ont.), a carboxylic acid cation exchange resin. Chelex-100™ has a very strong attraction for transition metals and hence is able to scavenge metal contaminants effectively from solutions (228).

The Chelex-100™ resin was initially suspended in 6M sodium hydroxide for 1 hour, then washed 10 times with 200 mL of double distilled/deionized water (ddH_2O) on a sintered glass funnel, the water being removed by filtration with suction. The resin was resuspended in 0.1M HCl, 300 mL, for one hour after which the acid was removed by again washing with ddH_2O. The exchanger was then packed into an acid-washed 2.5 x 40 cm glass column and washed thoroughly with acetate buffered saline (ABS), 0.1M, pH 7.4 until the pH of the effluent and influent was similar. All buffers and solutions were passed through the equilibrated column, collected in acid-washed containers, and stored at 4°C.

Reintroduction of significant metal contamination must be prevented once all the solutions have been purified. Therefore all glassware was acid-washed in a 1:1 mixture of concentrated HNO_3:H_2SO_4. In addition, syringes and other plastic materials used were soaked in 0.01M ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific, Ottawa, Ont.) for 24 hours and then thoroughly rinsed with ddH_2O.

2. Effect of Buffer Systems on ^{67}Ga-Deferoxamine Formation Using $^{67}GaCl_3$ and ^{67}Ga-Citrate

To determine whether a particular solvent system would affect the labeling of deferoxamine with gallium, 10.0 mg of deferoxamine mesylate, (Ciba-Geigy Canada Ltd, Mississauga, Ont.) was dissolved in 1.0 mL of each of the following:

1. citrate buffered saline (CBS)
   (0.1M citrate buffer in 0.15M sodium chloride, pH 7.4),
2. phosphate buffered saline (PBS)
   (0.1M phosphate buffer in 0.15M NaCl, pH 7.4), and
3. acetate buffered saline (ABS)
   (0.1M acetate buffer in 0.15M NaCl, pH 7.4).

All the buffers were prepared using ddH_2O purified with the Nanopure II system (Sybron/Barnstead) and passed through a Chelex-100™ column.

To each of the above deferoxamine solutions 5 MBq of either $^{67}GaCl_3$ (Merck-Frosst, Kirkland, Que.) or ^{67}Ga-citrate (Merck-Frosst) was added. The $^{67}GaCl_3$-deferoxamine solutions were allowed to incubate for 15 minutes, whereas ^{67}Ga-citrate-deferoxamine preparations were incubated for 1 hour. Aliquots of the ^{67}Ga-deferoxamine solutions were removed, spotted on Whatman #1 chromatography paper, and dried. The chromatograms were developed in 85% methanol and were allowed to run approximately 20 cm. The strips were cut into 1 cm segments and assayed for radioactivity in a Picker Spectroscalar 4R gamma spectrometer (Picker Nuclear, White Plains, New York). All chromatography was performed in duplicate.

3. Availability of Deferoxamine for Binding Metals

In order to determine whether all the deferoxamine in solution was available for binding with a metal, 100 μL (10 μmoles) of a 0.1M solution of ferric perchlorate (Alfa Inorganics, Danvers, Mass.) in ddH_2O was added to 10 μmoles of deferoxamine mesylate. After 15 minutes incubation, the resultant ferrioxamine complex was diluted to 3.0 mL with water and the pH measured with a PHM 62 Standard pH meter (Radiometer, Copenhagen, Denmark). At this point the absorbance of the golden coloured solution was measured at 430 nm against an appropriate blank (2.9 mL ddH$_2$O plus 100 μL iron perchlorate 0.1M) using a Gilford 250 Spectrophotometer (Gilford Instruments Lab. Inc., Oberlin, Ohio). The solution's absorbance was *again* measured at 1 hour and 3 hours after initial complexation of iron with deferoxamine. The experimental millimolar extinction coefficient was calculated using the formula:

$$A = \epsilon ct$$

where A is the absorbance of the solution, ϵ is the millimolar extinction coefficient, c is equal to the concentration in mmoles/L, and t is the pathlength of the u.v. cell (1 cm).

The availability of deferoxamine was determined as follows:

$$\text{\% deferoxamine available} = \frac{\text{experimental } \epsilon}{\text{published } \epsilon} \times 100$$

where the published millimolar extinction coefficient equals 2.5 (190).

The rate and extent of metal-deferoxamine formation at physiologic pH was also examined. A solution of 0.01M ferric perchlorate in 0.1M nitrilotriacetic acid (NTA) was prepared and the pH adjusted to 7.4 with 1N NaOH and ABS 0.1M pH 7.4. A 2.3 mM stock solution of deferoxamine was also prepared in 1 mL of ABS. Aliquots of the stock solution were removed to prepare a series of deferoxamine solutions ranging from 57 to 343 nmoles/mL. Ferric-NTA (100 μL, 1000 nmoles) was then added to 2.9 mL of the deferoxamine solutions and allowed to incubate for time periods from 15 minutes to 4.5 hours. The absorbance of the ferrioxamine complexes was read at 430 nm against a blank containing ferric-NTA in ABS. The millimolar extinction coefficient was again calculated and the percent of deferoxamine available determined.

4. Stability of Native Fibrinogen

To study the stability of a concentrated fibrinogen solution (i.e. a concentration which exceeds that found in the blood), a 6.8 mg/mL solution of human fibrinogen (Type IV, Sigma Chemical Co., St. Louis, Mo.) was prepared in 0.15M NaCl, pH 7.4. The fibrinogen was incubated in a water bath at 37°C, and 0.25 mL aliquots were chromatographed on a

Bio-Gel A-1.5m™ (Bio-Rad Lab. Ltd, Mississauga, Ont.) column at the following intervals: 15 minutes, 1, 2, 4, 6, and 19 hours. The elution profile, monitored at 280 nm, was recorded and compared to a profile obtained by chromatographing a 1 mg/mL fibrinogen solution (0.25 mL) applied 15 minutes and 2 hours after dissolution.

B. General Procedure for the Preparation of ^{67}Ga-Fibrinogen

Utilizing a glutaraldehyde two-step procedure (77), deferoxamine-fibrinogen was prepared by reacting deferoxamine-glutaraldehyde with human fibrinogen. The reaction was terminated by the addition of sodium borohydride. The mixture was subsequently purified by either dialysis or gel filtration. The purified product was then labeled with ^{67}Ga and the yield of ^{67}Ga-fibrinogen determined.

1. Preparation of Deferoxamine-Fibrinogen

Deferoxamine mesylate solutions ranging in concentration from 0.02M to 0.27M were prepared in ABS buffer (0.1M acetate, 0.15M NaCl, pH 7.4). Next, an appropriate volume of a 10% glutaraldehyde solution (Aldrich Chemical Co., Milwaukee, Wis.) was added such that the molar ratio of glutaraldehyde to deferoxamine was maintained at 0.83 moles glutaraldehyde:1 mole deferoxamine. After stirring this reaction mixture for 5 minutes at room temperature, 45 μL was added to 4.5 mL of a 0.02 mM or 0.03 mM solution of human fibrinogen previously prepared in ABS and incubated at 37C for 1 to 4 hours. The resultant mixture was stirred for 45 minutes at room temperature. Sodium borohydride in water (45 μL of a 1.8mg to 20 mg/mL solution) was prepared and immediately added to the deferoxamine-glutaraldehyde-fibrinogen complex. A constant ratio of 2.9 moles $NaBH_4$ per mole glutaraldehyde was maintained throughout the series of experiments. The mixture was then allowed to react for 10 minutes at room temperature after which time a purification step was initiated.

2. Purification of Deferoxamine-Fibrinogen

a. Dialysis

The entire protein-conjugate mixture was dialyzed either at 4°C or at room temperature for 24 hours against 2 L of ABS using Spectrapor™ dialysis membrane (Spectrum Medical Industries, Inc., Los Angeles, Ca). This particular membrane retained molecules greater than 12,000 to 14,000 daltons. Therefore, any unreacted deferoxamine-glutaraldehyde (approximate molecular weight of 660 daltons) would be removed from the deferoxamine-protein complex.

b. Gel Filtration

Bio-Gel P-6DG™ (Bio-Rad Lab. Ltd, Mississauga, Ont.) is a polyacrylamide gel with an exclusion limit of 6,000 daltons. Thus, modified fibrinogen molecules will be eluted in the void volume, with the unreacted reagents being retained by the gel.

Bio-Gel P-6DG Column Preparation:

The gel was allowed to hydrate for 30 minutes at room temperature in acetate buffered saline containing 0.02% sodium azide (MCB Reagents, Darmstadt, Germany) and 0.01M EDTA. The fine particles were removed by suctioning off the top layer of buffer. The volume was then readjusted to twice the expected packed bed volume (8 mL/gram Bio-Gel P-6DG) and degassed at reduced pressure for several hours. Two column lengths were prepared:

1. a 1.5 x 23 cm glass column containing a packed bed volume of approximately 40 mL, and
2. a 1.5 x 37 cm column, with a 65 mL bed volume.

The above columns were then equilibrated with ABS buffer.

Separation Procedure:

To prevent non-specific adsorption, 0.5 mL of a 1 mg/mL human fibrinogen solution in ABS was passed through the columns prior to purification of the protein-conjugate mixture. The protein complex (4.4 mL) was then carefully layered onto the bed surface and allowed to drain into the bed. The flow rate was adjusted to 35 mL per hour using an LKB (Bromma, Sweden) 2232 Microperpex S Peristaltic Pump. An LKB 2238 Uvicord S II photometer was set at 226 nm to detect the presence of both the fibrinogen complex and unreacted deferoxamine-glutaraldehyde and their optical densities were recorded using an LKB 2210 Two-Channel Recorder. The eluate was collected in 4.6 mL aliquots for further analysis.

3. Radiolabeling Procedure

Either 20 MBq or 40 MBq of ^{67}Ga-citrate, supplied by the Atomic Energy of Canada Ltd., Ottawa, Ont. (AECL), was added to 1.0 mL of the purified deferoxamine-fibrinogen fraction. The radioactive mixture was then allowed to incubate for 1 hour at room temperature before further analyses were performed.

C. Methods of Analysis of Deferoxamine-Fibrinogen

1. Determination of Deferoxamine-Fibrinogen Molar Ratio

a. Determination of Protein Concentration

The protein concentration of fibrinogen was determined by diluting 100 μL aliquots of the protein solution to 4.0 mL with acetate buffered saline 0.1M, pH 6.4. The optical density of the solution was measured at 280 nm (blank = ABS 0.1M, pH 6.4). An absorbance coefficient of $E^{1\%}_{1cm} = 1.55$ (229) was used to convert absorbance readings to concentration. Protein concentration was determined prior to, and after the reaction with deferoxamine-glutaraldehyde. Protein concentration was also determined for all fractions collected from the column during which a protein peak was observed.

b. Determination of Deferoxamine Concentration

A 100 μL aliquot of the original reaction mixture was diluted to 2.9 mL with ABS 0.1M, pH 7.4 and the deferoxamine concentration determined. In addition, of each fraction collected during gel filtration, 2.9 mL was tested for the presence of deferoxamine.

A series of 2.9 mL deferoxamine standards in ABS ranging from 57 to 343 nmoles/mL were prepared. To both the standards and the above samples, 100 μL of 0.01M ferric-nitrilotriacetic acid (Fe-NTA), prepared as described previously, was added, and the ferrioxamine complexes were allowed to incubate for 20 minutes. The absorbance of these solutions was measured at 430 nm against a blank containing buffer and Fe-NTA. A standard curve of optical density versus concentration was used to determine the concentration of deferoxamine in each fraction. The minimal detectable concentration of deferoxamine was also calculated by a method described by Currie (230). Knowing the concentrations of both deferoxamine and protein in the samples, the deferoxamine-fibrinogen molar ratio was calculated:

$$\text{molar ratio} = \frac{\text{nmoles/mL deferoxamine}}{\text{nmoles/mL fibrinogen}}$$

2. In Vitro Properties of Deferoxamine-Fibrinogen

a. Clottability

To examine the effect of linking fibrinogen with a bifunctional chelating agent, the clottability was measured according to a method described by Endres and Scherega (231).

The following solutions were prepared in advance and stored at 4°C: a 40% urea solution in 0.2N sodium hydroxide, and a 1.0M sodium bromide/0.05M sodium acetate solution, pH = 5.3. Thrombin (1000 NIH units per vial, Parke-Davis Canada Inc., Edmonton, Alta.) was reconstituted immediately prior to use with 4.0 mL water to give a final concentration of 250 units/mL.

The procedure for determining the clottability of fibrinogen was as follows: to each of the protein solutions prepared previously for the determination of fibrinogen concentration, 10 units (40 μL) of thrombin was added. The solution was allowed to clot at room temperature for at least 2 hours and then vortexed vigorously. The solution was then centrifuged at 3,500 rpm for 10 minutes to aggregate the clots. These clots were carefully removed with a glass pipette and transferred to 5 mL polystyrene culture tubes. One mL of the 40% urea solution was added to each tube and the clots were incubated in a 37°C water bath. Immediately after the clots had redissolved (approximately 5 minutes), 3.0 mL of the sodium bromide/sodium acetate solution was added, and these mixtures were allowed to incubate 10 minutes. The absorbance at 280 nm was measured against a blank containing urea and sodium bromide. The percent clottability was then determined by comparing the absorbances of the redissolved clots to the absorbances of the solutions prior to clotting:

$$\%\ \text{clottability} = \frac{\text{Absorbance of redissolved clot}}{\text{Absorbance of protein solution before clotting}} \times 100$$

b. SDS-Disc-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE allows biological molecules to be separated on the basis of size alone if the proteins are first solubilized with the detergent sodium dodecylsulfate (SDS) (232). Their molecular weights can be determined by comparing the proteins' electrophoretic mobility (R_f) to standard proteins of known molecular weights.

Reagents and Equipment:

The Protean™ Slab Cell (Bio-Rad Lab. Ltd., Mississauga, Ont.) is a vertical slab electrophoresis instrument suitable for most electrophoretic techniques including SDS-Electrophoresis. The cell accepts either one or two gel slabs (16 x 14 cm) per run and accommodates up to 15 samples per slab. An ABN-Bionuclear EPS-4000 power supply (American BioNuclear, Emeryville, Ca.) was connected to the slab cell and could be set to a constant current or constant voltage.

All the reagents needed for the preparation of electrophoresis buffers and gels were obtained from Bio-Rad Lab.

Preparation and Casting of Discontinuous Gels:

The gels were prepared according to the method described by Laemmli (233). Briefly, a separating gel was prepared first by pouring a 7.5% acrylamide-bis solution into a slab and allowing it to harden overnight at 4°C. A 4% acrylamide-bis stacking gel solution was then layered on top. A 'comb' was placed into the stacking solution prior to its polymerization so that once polymerized, and the comb removed, a series of wells were available for sample application.

Sample Preparation:

An aliquot of purified deferoxamine-fibrinogen fraction was diluted with ABS such that the final protein concentration was 1 mg/mL. Seventy-five μL of sample buffer (containing glycerol, 10% SDS, and 0.05% w/v bromophenol blue) was then added to a 25 μL aliquot of the 1 mg/mL protein solution, and 25 μL of this preparation was carefully layered onto the discontinuous gel.

Molecular weight markers were also prepared in the following manner: 95 μL of sample buffer was added to 5 μL of Molecular Weight Protein Standard for SDS-PAGE (Bio-Rad Lab. Ltd, Mississauga, Ont.), of which 25 μL was applied to the gel. A 6.25 μg sample (25 μL) of native fibrinogen in sample buffer was also applied to the gel for direct comparison with the fibrinogen conjugate.

Electrophoresis:

Two slabs were placed in the electrophoresis tank and a tris-glycine-SDS electrode buffer was added to both the upper and lower reservoirs. The lower electrode buffer was kept cool by passing water through the heat exchanger ports. In addition, the buffer was stirred constantly to keep the temperature uniform. A current of 50 mA was applied for 1 hour after which constant voltage was applied for 2.5 hours, or until the bromophenol blue tracking dye had migrated approximately 10 cm down the gel.

The gels were removed from the cell and stained with Coomassie Brilliant Blue R Stain for 1 hour and destained in 7% acetic acid/5% methanol. The R_f values were determined by comparing the migration distances of the blue protein bands and the bromophenol blue tracking dye:

$$R_f = \frac{\text{migration distance (mm) protein bands}}{\text{migration distance (mm) tracking dye}}$$

Using the R_f values obtained from the molecular weight markers, a standard curve of molecular weight (log) versus relative mobility was then prepared to determine the molecular weight of the deferoxamine-fibrinogen complex.

D. Methods of Analysis of ^{67}Ga-Deferoxamine-Fibrinogen

1. Yield of ^{67}Ga-Deferoxamine-Fibrinogen and Analysis of Non-Protein Bound Radioactivity

a. Gel Filtration

The Bio-Gel P-6DG columns and equipment previously described for purification of deferoxamine-fibrinogen were also utilized to determine the radiolabeling efficiency of the deferoxamine-fibrinogen complex.

Approximately 1 mL of ^{67}Ga-fibrinogen was applied to the column and the flow rate adjusted to 35 mL/h. A 226 nm u.v. filter was used to detect protein, deferoxamine, and citrate (from ^{67}Ga-citrate). The polyethylene tubing carrying the eluate was also passed across the face of a shielded 3 x 3 inch NaI(Tl) crystal detector attached to a single channel analyzer (Canberra Industries Inc., Meridan, Conn.) calibrated for the photopeaks of ^{67}Ga. Simultaneous detection of radioactivity and optical density was recorded with an LKB dual pen recorder.

The radioactivity elution profile provided information on the relative distribution of radioactivity between ^{67}Ga-fibrinogen and non-protein bound radioactivity (as ^{67}Ga-deferoxamine or ^{67}Ga-citrate). The amount of radioactivity in each fraction was determined using a CRC™-10R BC dose calibrator (Ohio Nuclear Ltd. Downsview, Ont.) These values were then expressed as a percentage of the total radioactivity initially applied to the column (after decay corrections were performed).

b. Paper Chromatography

An aliquot of ^{67}Ga-fibrinogen, prior to column chromatography, was spotted on Whatman #1 Chromatographic paper strips (1.5 x 20 cm). In addition, the radiolabeled protein fraction eluted from the Bio-Gel column, and a fraction containing non-protein bound activity were also applied onto chromatographic paper. The strips were dried and then developed in the following solvent systems:

1. n-butanol:n-propanol:water - 9:6:5 (57)
2. pyridine:95% ethanol:water - 1:2:4 (234)

With the butanol:propanol:water system, ^{67}Ga-citrate remains at the origin along with ^{67}Ga-fibrinogen, whereas the R_f of ^{67}Ga-deferoxamine approximates 0.5. In the pyridine:ethanol:water system, ^{67}Ga-fibrinogen again remains at the origin while the other ^{67}Ga-complexes travel to the solvent front.

After the solvents were allowed to ascend for 15 cm, the paper was removed, air dried, cut into 1 cm sections, and counted in a Beckman Gamma-8000 automatic gamma well-counter (Beckman Instruments Inc., Irvine, Ca.). The percentage of radioactivity in each peak was determined by dividing the sum of the radioactivity in the particular sections by the radioactivity on the entire strip.

c. Trichloracetic Acid (TCA) Precipitation

TCA 20% (1.0 mL) was added to a 100 μL aliquot of labeled protein which contained 10 μL of 25% HSA. The suspension of denatured protein was vortexed to ensure complete mixing of TCA with fibrinogen and then centrifuged at 2,000 rpm for 10 minutes. The total radioactivity was assayed before the supernatant was removed. The supernatant and precipitate were then assayed separately using the TN-7200 multichannel analyzer (Tracor Northern, Middleton, Wis.) linked to a NaI(Tl) crystal.

d. Paper Electrophoresis

One μL aliquots of ^{67}Ga-fibrinogen, ^{67}Ga-citrate, and freshly prepared ^{67}Ga-deferoxamine were spotted onto the centre of 2 cm x 30 cm strips of Whatman 3MM paper, previously saturated with 0.025M tris/0.05M barbital buffer pH 8.8. The strips were placed in an electrophoresis chamber (Gelman Sciences Inc, Montreal, Que.) connected to a power supply, and constant voltage of 250V was applied for 3 hours. The paper strips were then removed, air dried, and cut into 1 cm segments for counting in a Beckman Gamma-8000 gamma spectrometer. One cm sections of Whatman 3MM paper were spotted with equivalent volumes of the samples and counted as standards to ensure that all radioactivity had remained on the strips.

In this system, ^{67}Ga-fibrinogen remains at the origin, ^{67}Ga-deferoxamine moves approximately 6 cm toward the cathode, and ^{67}Ga-citrate travels about 3 cm toward the anode. The percentage of activity in each peak was determined by dividing the radioactivity in the peaks by the radioactivity in the standard.

e. SDS-PAGE

SDS-polyacrylamide gel electrophoresis was employed to determine the yield of ^{67}Ga-fibrinogen and to determine, if possible, the radiochemical nature of the free radioactivity in the labeled product. The methodology described earlier was used for both gel and sample preparations. Twenty-five μL aliquots of the following were layered onto a discontinous gel: ^{67}Ga-fibrinogen, ^{67}Ga-citrate, and ^{67}Ga-deferoxamine (assays of 20 MBq/mL).

The radioactive gel slab was run alongside the gel containing non-radiolabeled deferoxamine-fibrinogen. Prior to application of a current to the cell, 1.0 mL aliquots of electrode buffer were removed from the upper and lower reservoirs. After a total running time of 3.5 hours, the gels were removed, and 1.0 mL aliquots of the electrode buffer were again taken. The slab containing radioactivity was cut into 1 cm segments and assayed for radioactivity in a Beckman Gamma-8000 counter. The electrode buffer samples were also counted along with 25 μL aliquots of those samples applied to the gel (to ensure that all the radioactivity had remained on the gel).

Since both the radioactive and non-radioactive gels were run together, the R_f values of both the stained protein and radiolabeled protein would be equal. Therefore the percent labeling efficiency of ^{67}Ga-fibrinogen was calculated by dividing the activity of the radiolabeled protein peak by the amount of ^{67}Ga-fibrinogen radioactivity applied. Other peaks found in the lane containing ^{67}Ga-fibrinogen were compared to the R_f values obtained for ^{67}Ga-citrate and ^{67}Ga-deferoxamine.

2. Properties of ^{67}Ga-Fibrinogen

a. Gel Permeation Chromatography

Bio-Gel A-1.5m™ (Bio-Rad Lab. Ltd., Mississauga, Ont.) is an agarose gel which is suitable for separating aggregates, proteins and polypeptides since its fractionation range lies between 10,000 and 1,500,000 daltons. The procedure for the preparation of a column with a bed volume of 78 mL was essentially the same as for Bio-Gel P-6DG except that hydration was not necessary, i.e. Bio-Gel A-1.5m is supplied fully-hydrated.

^{67}Ga-fibrinogen (0.25 mL) was layered onto the bed followed by ABS buffer. The flow rate was adjusted to 10 mL/h and the radioactivity and absorbance elution profiles were recorded using an LKB Two-Channel Recorder. The column was allowed to flow until all the radioactivity applied (approximately 2 MBq) was retrieved. The u.v. elution profile was then compared to profiles obtained from passing 0.25 mg of native fibrinogen through the column.

b. Fraction of Radiogallium in the Clot (Isotopic Clottability)

To 1.0 mL of radiolabeled fibrinogen, 3.0 mL of ABS 0.1M, pH 6.4 was added. The total radioactivity in the tube was assayed in a dose calibrator. The solution was then clotted, vortexed, and centrifuged as described for determining the clottability of non-radiolabeled deferoxamine-fibrinogen. The clot was quantitatively transferred onto a Whatman glass microfibre filter previously placed on a Millipore fritted glass filter support (Millipore Ltd., Mississauga, Ont.) Excess supernatant within the clot was removed by suction and the dehydrated clot transferred to a polystyrene culture tube. The radioactivity in the clot, supernatant and filter paper was then measured. The percent isotopic clottability was determined by dividing the radioactivity remaining in the clot by the total radioactivity prior to clotting.

c. Stability of the Label

To establish whether ^{67}Ga remained complexed with deferoxamine-fibrinogen, a 1.0 mL sample of the radiolabeled fibrinogen fraction was reapplied to the Bio-Gel P-6DG column 6 and 24 hours after initial preparation of ^{67}Ga-fibrinogen. Fractions were again collected and each fraction was assayed for activity and compared to the amount of radioactivity applied to the column. Radioactivity and u.v. elution profiles were recorded as well.

E. Determination of Metal Contamination in ^{67}Ga-Citrate

Since metal contamination could seriously affect the ability of deferoxamine to complex radiogallium (183), four samples of ^{67}Ga-citrate (Merck-Frosst and AECL) were analyzed by neutron activation analysis for the presence of zinc and iron.

Standards of 5, 10, 25, 50, and 100 μg of zinc (0.998 μg/μL, Aldrich Chemical Co., Milwaukee, Wis.), and 10, 25, 50, 75, and 100 μg of iron (1.004 μg/μL, Aldrich) were spotted on Whatman #1 filter paper discs. The following decayed ^{67}Ga-citrate samples were similarly prepared:

1. Sample A - Lot 79220-1, Merck-Frosst, 425 μL,
2. Sample B - Lot 79266-1, Merck-Frosst, 650 μL,
3. Sample C - Lot BGa126, AECL, 450 μL, and
4. Sample D - Lot 80772-5, Merck-Frosst, 800 μL.

The standards and samples were sealed in polyethylene envelopes and subjected to a 4 hour irradiation, at a flux of 1 x 10^{12} n·cm^{-2}s^{-1} along with blank filter paper discs, in the University of Alberta Slowpoke Facility Reactor. To reduce background radioactivity caused by the irradiation of sodium and chlorine, the samples were allowed to decay for a 3 week period along with the standards. The samples were then counted in a lead cave for 9 hours using a 413R closed end coaxial Ge(Li) Spectrometer coupled to a Series-80 Multichannel

Analyzer (Canberra Industries Inc., Meridan, Conn.). The gamma spectra of the ^{59}Fe and ^{65}Zn standards were then compared with the sample spectra and quantitated.

IV. RESULTS AND DISCUSSION

A. Preliminary Work

1. Effect of Buffer Systems on ^{67}Ga-Deferoxamine Formation Using ^{67}GaCl$_3$ and ^{67}Ga-Citrate

It is known that the gallic ion is capable of forming a variety of ionic species in aqueous media, the formation of these species being dependant on pH, the solvent system used, and the presence of complexing or chelating agents (195,234). Therefore it was necessary to ascertain which buffer system would provide the best medium for the complexation of radiogallium with deferoxamine using either ^{67}GaCl$_3$ or ^{67}Ga-citrate.

A paper chromatography system employing 85% methanol was used to determine the formation of ^{67}Ga-deferoxamine. In methanol, ^{67}Ga-deferoxamine migrated to the solvent front, and ^{67}Ga-citrate achieved an R_f of approximately 0.5. Radioactivity remaining at the origin was due to the formation of insoluble hydroxide (234). The results of paper chromatography are presented in Table 4.

Approximately 83% of the total radioactivity added as ^{67}GaCl$_3$ resulted in a ^{67}Ga-deferoxamine complex in citrate buffered saline (CBS), compared to 86% ^{67}Ga-deferoxamine formation in phosphate buffered saline (PBS), and 70% in acetate buffered saline (ABS). In contrast, 75% of the total radioactivity occurred as ^{67}Ga-deferoxamine when ^{67}Ga-citrate and CBS were employed, versus 84% for ^{67}Ga-deferoxamine in PBS, and 89% in ABS.

In the case of deferoxamine dissolved in CBS, the unreacted radioactivity was observed to behave as ^{67}Ga-citrate, regardless of whether ^{67}GaCl$_3$ or ^{67}Ga-citrate was used. Although ^{67}Ga-deferoxamine has a formation constant greater than ferrioxamine (60) ($\log K > 31$) versus a log stability constant of 10.02 for ^{67}Ga-citrate (59), the excessive amount of citrate from the buffer, and from ^{67}Ga-citrate (when employed as the radiolabeling agent) could effectively compete with deferoxamine for the radiogallium. Also, the transfer of ^{67}Ga from citrate to deferoxamine would be retarded, which may account for the lower ^{67}Ga-deferoxamine yield observed when ^{67}Ga-citrate, rather than ^{67}GaCl$_3$, was used.

Table 4

Effect of Various Buffer Systems on ^{67}Ga-Deferoxamine Formation[1] Using ^{67}GaCl$_3$ and ^{67}Ga-Citrate[2]

Sample	Percent Activity as ^{67}Ga-Deferoxamine[3]	
	^{67}GaCl$_3$[4]	^{67}Ga-Citrate[5]
Deferoxamine in CBS	82.6 (82.5-82.7)	75.2 (73.6-76.8)
Deferoxamine in PBS	85.8 (85.1-86.5)	84.1 (83.3-84.9)
Deferoxamine in ABS	69.6 (67.3-71.9)	88.9 (88.5-89.3)

[1] values expressed as the mean percent and (range) of duplicate determinations

[2] 5 MBq of radiogallium added to deferoxamine

[3] remaining radioactivity as ^{67}Ga-citrate or ^{67}Ga(OH)$_3$

[4] incubation time with deferoxamine: 15 minutes

[5] incubation time with deferoxamine: 1 hour

When deferoxamine was dissolved in PBS, 84 - 86% of ^{67}Ga-deferoxamine was formed. In this case, the remaining radioactivity was spread between the origin and R_f 0.7. The unbound radioactivity was likely a gallium hydroxide species (radioactivity at the origin), or another gallium species such as $Ga(OH)_4^{-1}$ which tends to form in an aqueous environment at neutral pH (234). In addition, phosphate anions are also capable of complexing with gallium (59). Thus this may account for the streaking observed in paper chromatography. With deferoxamine dissolved in ABS, only 70% of the total radioactivity was in the form of ^{67}Ga-deferoxamine when $^{67}GaCl_3$ was utilized. The remaining radioactivity stayed at the origin. In contrast, when using ^{67}Ga-citrate, 89% of the radioactivity was bound to deferoxamine with the remaining 10% behaving as ^{67}Ga-citrate. Acetate anion is a much weaker complexing agent than citrate (184) and would not likely form a ^{67}Ga-acetate complex easily, especially when other stronger complexing agents are available for binding. Therefore, since:

1. a physiologic pH should be employed when 'working' with biological molecules such as fibrinogen,
2. there is an increased risk of forming insoluble hydroxides and other gallium species when using $^{67}GaCl_3$ to prepare a radiogallium-deferoxamine complex at neutral pH,
3. excess citrate could compete with deferoxamine for ^{67}Ga, and
4. ^{67}Ga may have a higher affinity for phosphate ions than for acetate ions,

^{67}Ga-citrate was chosen as the radiolabeling agent, and acetate buffered saline 0.1M, pH 7.4 was employed as the medium for ^{67}Ga-fibrinogen.

2. Availability of Deferoxamine For Binding Metals

During the course of their studies comparing uptake of gallium with iron in siderophore-transport systems, Emery and Hoffer developed a spectrophotometric assay for the quantitation of deferoxamine in solution (190). When deferoxamine and iron are mixed together, iron is rapidly complexed with deferoxamine to form ferrioxamine (58), a golden-brown coloured solution with an absorbance maximum at 430 nm (millimolar extinction of 2.5) (190).

This assay was applied to determine whether the total amount of deferoxamine added to fibrinogen was actually available for binding with gallium (i.e. was commercial deferoxamine free of contaminating metals such as iron and zinc?). When 10 μmoles of iron as ferric perchlorate was added to 10 μmoles of deferoxamine in water (pH less than 3.5), an average of 88.7% (range=86.9% to 90.5%, n=3) of the deferoxamine was available for binding with iron within 15 minutes. However when an excess of iron (1000 nmoles), in the form of ferric nitrilotriacetic acid at neutral pH, was added to increasing concentrations of deferoxamine (57 to 343 nmoles), 111.8% (range=90.6% to 129.5%, n=12) of the deferoxamine formed a ferrioxamine complex within 20 minutes. The absorbance values measured at 430 nm for the detection of ferrioxamine did not change between 20 minutes and 4.5 hours indicating that the transfer of iron from nitrilotriacetic acid to deferoxamine was complete within 20 minutes.

It is possible that extraneous metal contaminants do exist in the deferoxamine product, since only 88.7% of the deferoxamine was complexed when equimolar amounts of iron and deferoxamine were used, and that at high iron concentrations these metals would be displaced from the deferoxamine molecules to form ferrioxamine. However, taking into account errors in weighing, pipetting, absorbance measurements, and the possibility of metal contaminants in the iron perchlorate solution itself, the amount of deferoxamine available for binding metals appears to be essentially equal to the total amount of deferoxamine in solution.

3. Stability of Native Fibrinogen

Fibrinogen is an unusually delicate, easily denatured molecule (124). Its concentration ranges from 2 - 4 g/L in plasma. At this concentration lyophilized fibrinogen will dissolve in buffer within 1 hour. However at higher concentrations, such as those employed for the preparation of deferoxamine-fibrinogen (6.8 - 10.2 mg/mL), the time for complete dissolution ranged from 1 to 4 hours. Therefore, an experiment was performed to determine whether any changes to the protein occurred over time. Aliquots of a 6.8 mg/mL fibrinogen solution were applied to a Bio-Gel A-1.5m column at specific intervals after dissolution, and optical density was monitored at 280 nm. The u.v. elution profiles were then compared to profiles of a 1 mg/mL solution of fibrinogen, aliquots of which were applied 15 minutes and 2 hours after dissolution.

The difference in the elution profiles between a 1 mg/mL fibrinogen solution (Figure 4) and a 6.8 mg/mL solution (Figure 5) is well illustrated. At a low fibrinogen concentration only a small aggregate peak (elution volume of 30 mL) was observed after 15 minutes of incubation, as seen in Figure 4A. After a 2 hour incubation interval a much higher aggregate peak was observed (Figure 4B), however a fibrinogen peak remained evident. In contrast, the profiles of a 6.8 mg/mL fibrinogen solution indicated that a large amount of aggregation was present even 15 minutes after dissolution of the protein (Figure 5A). The profile did not change significantly between 15 minutes and 2 hours. However, between 4 and 19 hours the fibrinogen profile changed dramatically (Figures 5B, 5C, and 5D) with several shoulders in the elution profile becoming evident.

Therefore, it is apparent that when concentrated fibrinogen solutions were prepared, aggregate formation and perhaps polymerization of fibrinogen occurred after extended periods of time. Thus it was necessary to prepare deferoxamine-fibrinogen, label this complex with ^{67}Ga and analyze the radiolabeled product as quickly as possible to reduce the risk of denaturation inherent with fibrinogen itself.

B. Preparation of Deferoxamine-Fibrinogen

In the preparation of deferoxamine-fibrinogen, glutaraldehyde was first linked with deferoxamine via one of its aldehyde groups. This complex was then coupled to fibrinogen with the remaining free aldehyde group (as illustrated in Figure 6). This method of binding a bifunctional chelating agent to a protein is defined as a 'glutaraldehyde two-step method' developed by Avrameas and Ternynck (219). The purpose of employing two steps in producing a protein-chelate complex is to reduce the risk of protein-protein coupling with a homocoupling agent such as glutaraldehyde. The success of this and any other coupling method depends on the specific reaction conditions: ratio of chelating agent to protein,

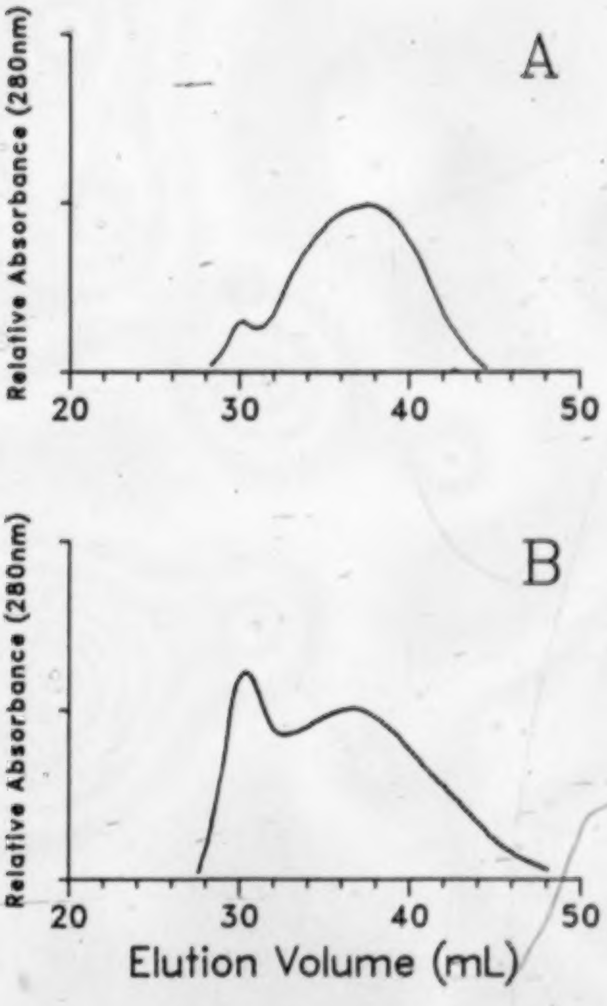


Figure 4: Elution Profiles of Human Fibrinogen (1mg/mL) after Incubation Time of (A) 15 minutes and (B) 2 hours

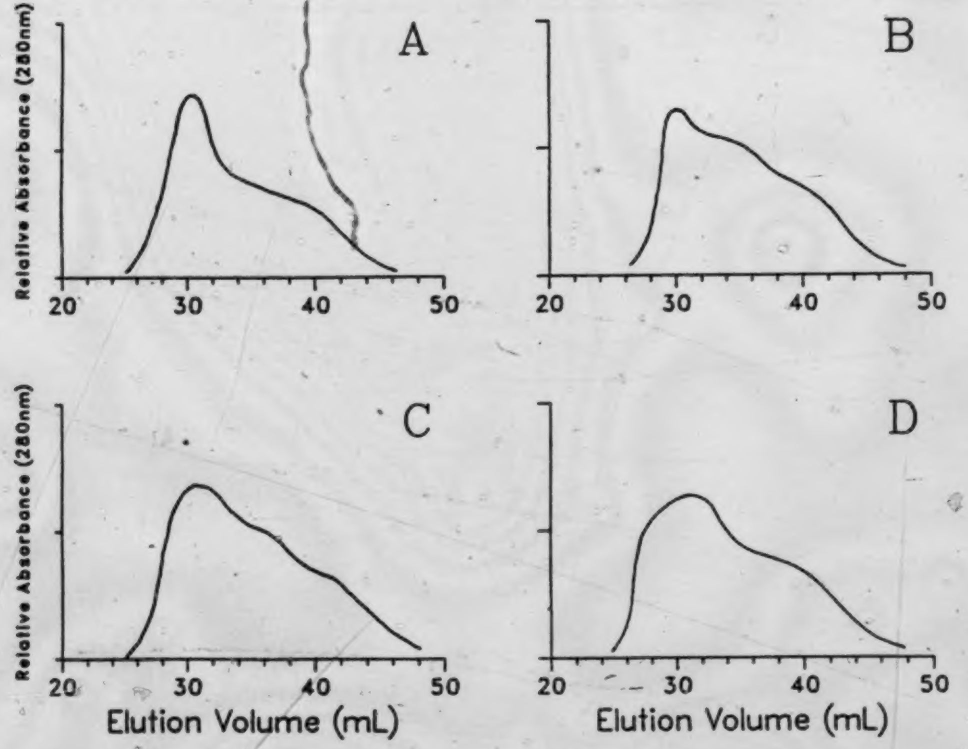


Figure 5: Elution Profiles of Human Fibrinogen (6.8mg/mL) after Incubation Time of (A) 15 minutes, 1 and 2 hours, (B) 4 hours, (C) 6 hours, and (D) 19 hours

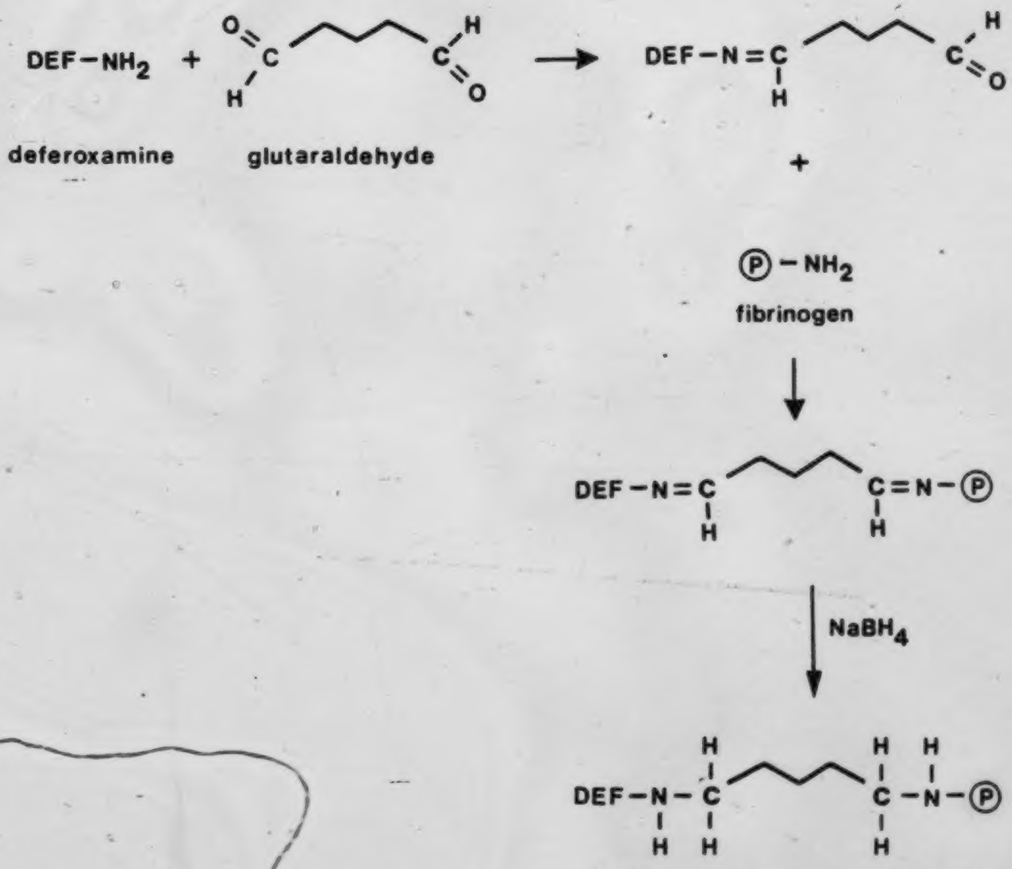

DEF−NH2
deferoxamine
glutaraldehyde
DEF−N=C
(P)−NH2
fibrinogen
C=N−(P)
NaBH4
DEF−N−C
C−N−(P)

Figure 6: Preparation of Deferoxamine-Fibrinogen

concentration, pH, reaction time, and temperature (203).

When working with a fragile protein such as fibrinogen, the pH of the reaction is already predetermined since an acidic or basic pH could seriously compromise the protein's biological integrity (132). Therefore a physiologic pH of 7.4 was maintained throughout the reaction between deferoxamine-glutaraldehyde and fibrinogen, and during purification of deferoxamine-fibrinogen.

Keeping the temperature low (approx. 4°C) should reduce the rate of protein denaturation. However at low temperatures, the solubility of fibrinogen decreases and precipitation occurs (235). Preliminary experiments, where the temperature was maintained at 4°C throughout the time of reaction, verified this fact; therefore, further experiments were performed at room temperature.

Wensel and Meares (172) have suggested that both the reagent and protein should be as concentrated as possible in order to drive the conjugation reaction to completion. Therefore, Ohmomo *et al* (77) prepared a 4.0 x 10^{-5}M solution of fibrinogen to complex with deferoxamine. However, difficulties were encountered in preparing such a concentrated fibrinogen solution; thus a protein concentration of only 6.8 mg/mL (or 2 x 10^{-5}M) was used. Even at this lower concentration, up to 4 hours were often required for complete dissolution of the protein.

The remaining reaction conditions, such as the amount of deferoxamine-glutaraldehyde reacting with fibrinogen, and the total reaction time, were similar to the work performed by Ohmomo (77) although other deferoxamine-fibrinogen ratios were also attempted to determine if the ratio employed by the Japanese researchers was, in fact, the optimum level of conjugation.

To complete the reaction, sodium borohydride was added to deferoxamine-glutaraldehyde-fibrinogen. At the concentrations used, sodium borohydride only reduces the imine bonds formed between deferoxamine and glutaraldehyde, and also between glutaraldehyde and fibrinogen leaving the important disulfide bonds intact (236).

C. Comparison of Dialysis and Gel Filtration Techniques for Purification of Conjugated Fibrinogen

Both dialysis and gel filtration are commonly employed methods in which materials are separated primarily on the basis of differences in molecular size and shape (223,237). Therefore both techniques were utilized in the purification of deferoxamine-glutaraldehyde-fibrinogen to evaluate the effectiveness of each technique in the removal of unreacted deferoxamine, glutaraldehyde, and deferoxamine-glutaraldehyde from the protein complex. Figure 7 illustrates the reaction conditions used to prepare deferoxamine-fibrinogen, the purification steps, and the analyses carried out for each purified product. The results comparing purification by dialysis and gel filtration are given in Table 5.

1. Protein and Deferoxamine Concentrations

When comparing the techniques of dialysis versus gel filtration with respect to protein and deferoxamine concentrations, dialysis consistently produced higher values. For example, the protein and deferoxamine concentrations averaged 15.7 nmoles/mL and 99.5 nmoles/mL respectively when dialysis was employed. In contrast, with gel filtration, only 10.3 nmoles/mL and 60.8 nmoles/mL were found for fibrinogen and deferoxamine respectively. This was due to dilution of the protein complex and must be expected even when large sample volumes are used during gel filtration.

2. Deferoxamine:Fibrinogen Molar Ratio

Although protein and deferoxamine concentrations were higher with dialysis than for gel filtration, the final molar ratios i.e. concentration of deferoxamine divided by protein concentration, were not statistically different. With dialysis the average ratio obtained was 6.3 moles deferoxamine per mole fibrinogen, whereas the ratio after gel filtration was calculated to be 6.1:1. These ratios coincide with those values reported by Ohmomo *et al* (77) who determined the conjugation level to range from 3 to 6 molecules of deferoxamine per molecule of fibrinogen.

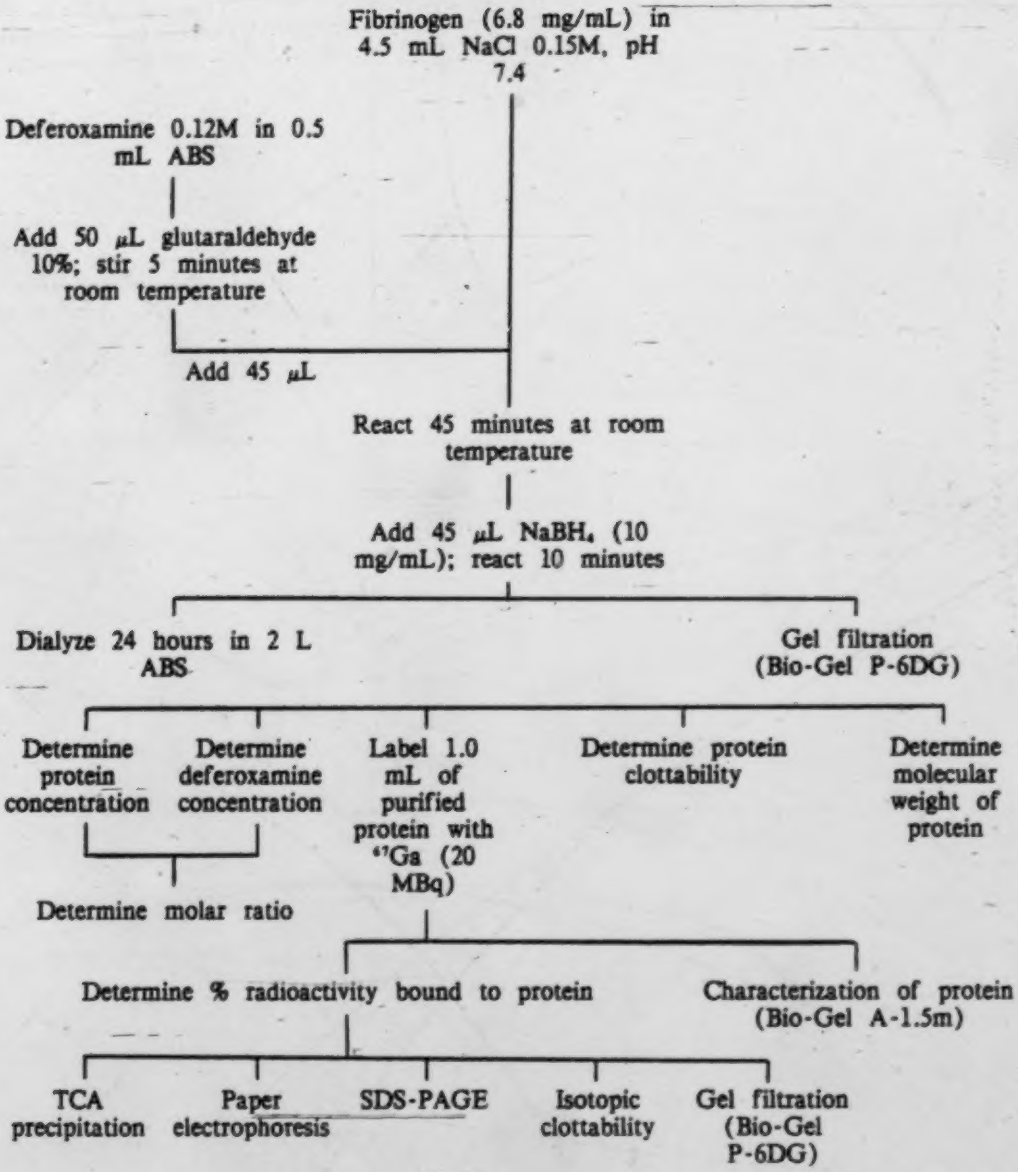

Fibrinogen (6.8 mg/mL) in 4.5 mL NaCl 0.15M, pH 7.4
Deferoxamine 0.12M in 0.5 mL ABS
Add 50 μL glutaraldehyde 10%; stir 5 minutes at room temperature
Add 45 μL
React 45 minutes at room temperature
Add 45 μL $NaBH_4$ (10 mg/mL); react 10 minutes
Dialyze 24 hours in 2 L ABS
Gel filtration (Bio-Gel P-6DG)
Determine protein concentration
Determine deferoxamine concentration
Label 1.0 mL of purified protein with ^{67}Ga (20 MBq)
Determine protein clottability
Determine molecular weight of protein
Determine molar ratio
Determine % radioactivity bound to protein
Characterization of protein (Bio-Gel A-1.5m)
TCA precipitation
Paper electrophoresis
SDS-PAGE
Isotopic clottability
Gel filtration (Bio-Gel P-6DG)

Figure 7: Flow Chart Comparing Dialysis and Gel Filtration Techniques in the Purification of Deferoxamine-Fibrinogen

Table-5

Comparison of Dialysis and Gel Filtration Techniques in the Purification of Deferoxamine-Fibrinogen[1]

	Dialysis[2]	Gel Filtration[2]
Protein Concentration (nmoles/mL)	15.7 (13.6-16.6)	10.3 (8.1-14.2)
Deferoxamine concentration (nmoles/mL)	99.5 (91.5-103.8)	60.8 (43.0-74.5)
Deferoxamine:fibrinogen molar ratio	6.3:1 (6.1-6.7:1)	6.1:1 (4.5-8.6:1)
Clottability (%)	74.4 (69.7-77.1)	75.6 (62.4-88.7)
Isotopic clottability (%)	17.5 (12.5-23.8)	18.8 (13.6-23.5)
Radiolabeling efficiency (%)		
(a) SDS-PAGE	13.6 (9.6-19.6)	27.4 (9.4-53.4)
(b)Bio-Gel P-6DG	28.3 (25.8-31.1)	30.9 (25.8-36.2)
Molecular weight[3] (daltons)	275,016 (273,588-277,103)	279,792 (270,283-281,222)

[1] deferoxamine-glutaraldehyde ratio constant at 0.83 moles glutaraldehyde per mole deferoxamine for the preparation of deferoxamine-fibrinogen

[2] mean and (range) of three determinations

[3] molecular weight of native fibrinogen estimated to be 284,637 daltons using SDS-PAGE

3. Percent Clottability and Isotopic Clottability

Using the unpaired t-test, no statistical differences were observed in either the clottability of fibrinogen, nor in the relative amount of radiogallium contained within the clot (isotopic clottability) when the two purification methods were compared. The mean values for percent clottability were 74.4% with dialysis versus 75.6% with gel filtration. These values compare well with the clottability achieved by clotting native fibrinogen under similar conditions (79.8 ± 1.5%, n=17). Therefore, the reaction conditions to which fibrinogen was exposed did not severely affect the protein's biological activity (as measured by clottability).

Isotopic clottability of ^{67}Ga-fibrinogen was quite low with an average of 17.5% (by dialysis) and 18.8% (with column chromatography) of the total radioactivity added becoming incorporated into the clot itself. However, the Japanese researchers (77) achieved an isotopic clottability of 85.3 ± 2.4%, (n=10). Ohmomo employed a method which measured the radioactivity of clottable ^{67}Ga-fibrinogen along with any radioactive supernatant trapped within the clot. In contrast, the procedure utilized in the present research project for determining isotopic clottability measured only the radioactivity in the dehydrated clot. Thus the different methodologies employed may have contributed to the variations observed between the values of isotopic clottability reported.

4. Percent Radiolabeling Efficiency

No difference in the percent radiolabeling efficiency as determined by column chromatography was observed between the products purified by dialysis (28.3%) or by gel filtration (30.9%). These values were, however, generally higher than those obtained with SDS-polyacrylamide gel electrophoresis (SDS-PAGE): 13.6% with dialysis and 27.4% with gel chromatography. It has been suggested by Ogsten (238) that complexes of ionic species (in cases where the mobilities of the complexes and the components are different from one another) will dissociate during an electrophoretic separation to an extent proportional to the square of the voltage gradient. Consequently, gallium which forms a coordinate-covalent bond with deferoxamine, may be pulled from the deferoxamine-fibrinogen complex during electrophoresis.

Column chromatography effectively separated ^{67}Ga-fibrinogen (elution volume, Ve of 16 mL) from both ^{67}Ga-citrate and ^{67}Ga-deferoxamine (Ve of 31 mL) as depicted in Figure 8, but could not separate the latter two products. However in SDS-PAGE, the remaining non-protein bound radioactivity behaved similarly to ^{67}Ga-citrate, travelling toward the anode along with the tracking dye. Therefore, the free radioactivity is most probably ^{67}Ga-citrate or some other unidentified ^{67}Ga species behaving like ^{67}Ga-citrate in SDS-PAGE. It is interesting to note that Ohmomo *et al* (77) also observed a second radioactive peak which displayed characteristics of neither labeled protein nor ^{67}Ga-deferoxamine chelates, but of some other unidentified ^{67}Ga-complex of low molecular weight.

5. Molecular Weight Estimation

SDS-PAGE was also employed to determine whether manipulation of fibrinogen by the addition of deferoxamine-glutaraldehyde would alter the protein's natural conformation in such a way that large polymer formation or perhaps deteriorative products of fibrinogen would be produced (239). Thus deferoxamine-fibrinogen was run on a 7.5% discontinuous gel along with an aliquot of native fibrinogen and molecular weight markers. The molecular weights listed in Table 5 are very similar for the compounds purified by both dialysis and column chromatography (≈275,000 and 280,000 daltons respectively), and compare well with the experimental value for native fibrinogen (285,000 daltons). Although these values were slightly lower than the generally accepted value of 340,000 ± 20,000 daltons (126), one may conclude that no major alterations occurred with fibrinogen when deferoxamine was added, since the electrophoretic mobility of deferoxamine-fibrinogen compared well with its native counterpart.

6. Characterization of ^{67}Ga-Fibrinogen

As with SDS-PAGE, Bio-Gel A-1.5m was used to determine, qualitatively, if the fibrinogen structure had been altered due to the addition of deferoxamine. The resultant u.v. and

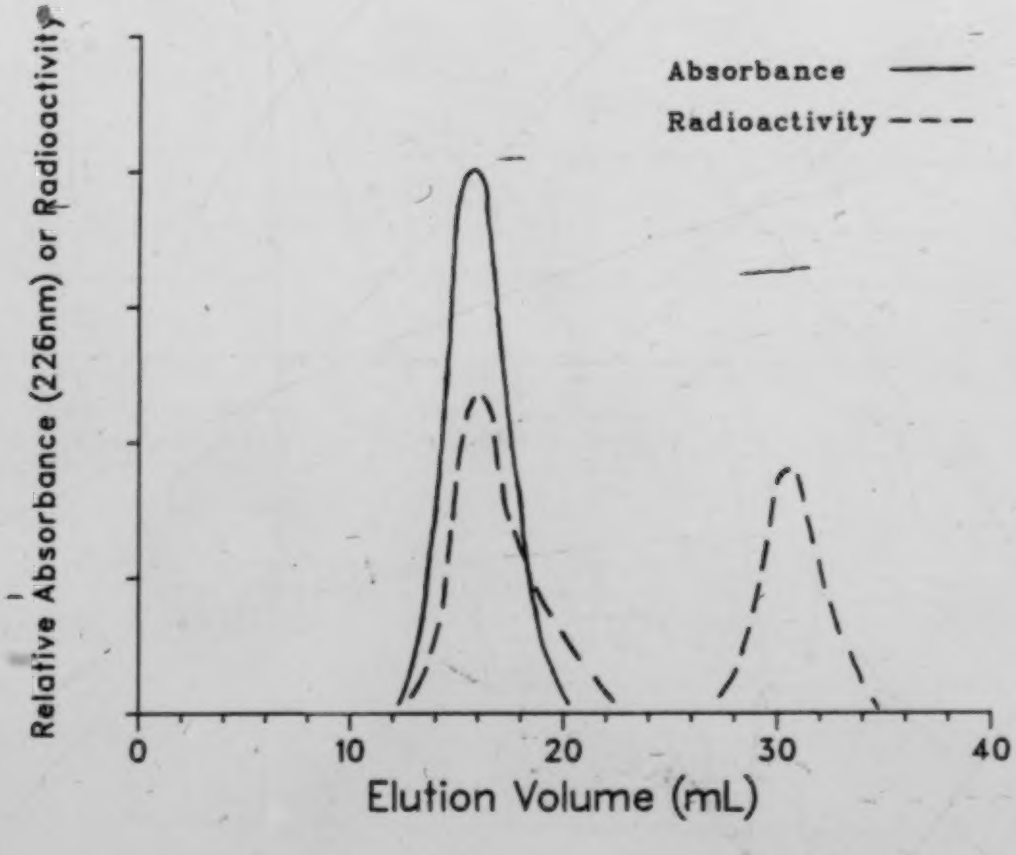


Figure 8: Analysis of ^{67}Ga-Fibrinogen by Bio-Gel P-6DG Column Chromatography

radioactivity elution profiles, as illustrated in Figure 9, mimic that profile of native fibrinogen which had been allowed to stand 2 to 4 hours after dissolution. Since approximately 4 hours are required to elute the protein fraction from the Bio-Gel A 1.5m column, polymerization of the protein may be occurring as the fibrinogen complex is passing through the column. Thus Bio-Gel A-1.5m gel filtration was not found to be useful in the qualitative analysis of radiolabeled deferoxamine-fibrinogen, due to the length of time required to elute the complex and the non-protein bound radioactivity from the column.

7. Paper Electrophoresis

This technique, adapted from the work of Schukla *et al* (240), was intended to determine the nature of the compounds present in the product. Preliminary experiments indicated that fibrinogen remained at the origin, ^{67}Ga-citrate moved approximately 3 cm toward the anode, and ^{67}Ga-deferoxamine travelled 6 cm toward the cathode. However, during analysis of ^{67}Ga-fibrinogen streaking was frequently observed between the origin and the anode. Although ^{67}Ga-deferoxamine was not observed, unequivocal information as to the nature of the compound running toward the anode could not be ascertained even when ^{67}Ga-fibrinogen was compared with a ^{67}Ga-citrate control. Hence paper electrophoresis was not utilized for further analysis of ^{67}Ga-fibrinogen preparations.

8. Trichloracetic Acid (TCA) Precipitation

TCA precipitation is a method often employed to determine the radiolabeling efficiency of iodinated proteins. Therefore, an attempt was made to use this analytical method to determine the radiolabeling efficiency of several ^{67}Ga-fibrinogen preparations. The amount of radioactivity precipitating along with the protein complex was consistently less than 1%. These results contrasted sharply with radiolabeling efficiency results obtained by gel filtration. Harwig *et al* (74) also found that precipitation with TCA gave erratic, unreliable results when determining the radiolabeling efficiency of ^{99m}Tc-fibrinogen.

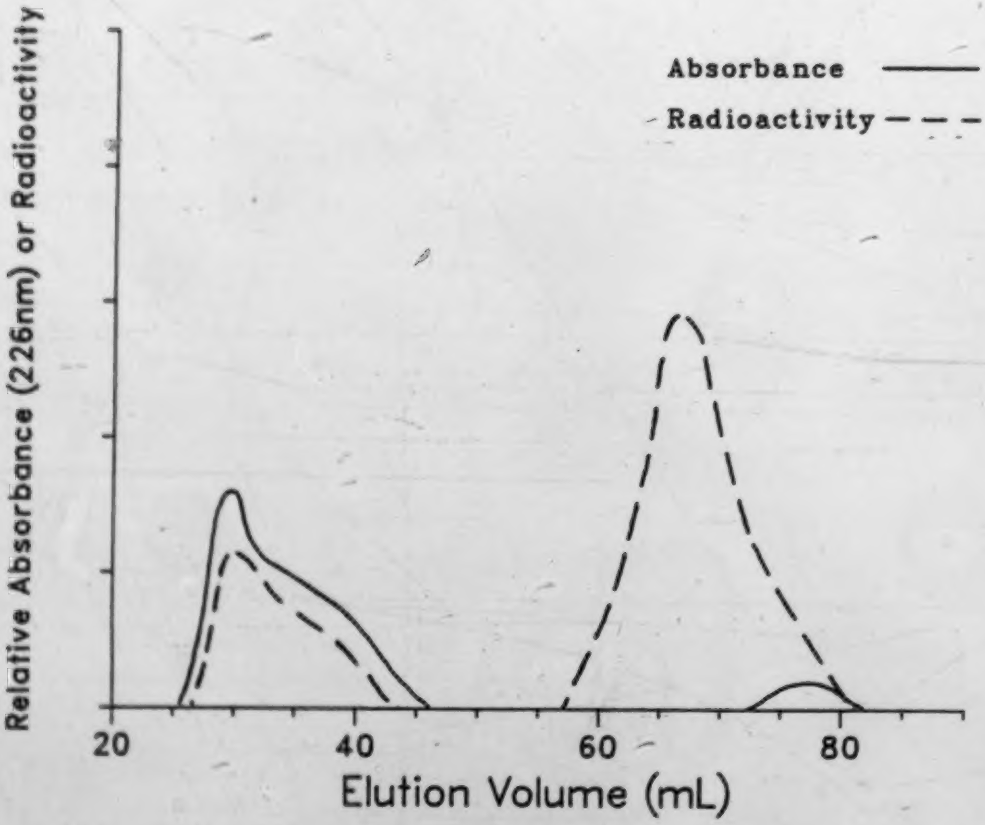


Figure 9: Characterization of ^{67}Ga-Fibrinogen by Bio-Gel A-1.5m Column Chromatography

It may be possible that the coordinate-covalent bond linking radiogallium with deferoxamine becomes weakened due to the strong acidic nature of TCA. Therefore ^{67}Ga would be released from the deferoxamine-fibrinogen complex, indicating extremely low yields of ^{67}Ga-fibrinogen. Because the TCA precipitation method yielded results which were inconsistent with those of gel filtration, TCA precipitation was not employed in later experiments.

To summarize the above, no statistical difference was observed in the ^{67}Ga-fibrinogen preparation when employing either dialysis or gel filtration as a purification step. Dialysis had some advantages in that the concentration of the protein complex was not altered during purification. Also, less variation occurred between results. For example, although the average clottability between the two purification methods differed by less than two percent, a range of 62.4% to 88.7% was found with gel chromatography versus a range of only 69.7% to 77.1% with dialysis. One disadvantage of using dialysis is the amount of time required to complete the purification step. At least twenty-four hours are necessary to remove the unreacted materials as compared to gel filtration which requires only 45 minutes. Although biological properties such as clottability did not seem to be greatly affected over a 24 hour period, the risk of aggregate formation exists, as was demonstrated when employing a Bio-Gel A-1.5m chromatography column.

Although sample dilution occurs during gel filtration, Bio-Gel P-6DG provides rapid results with quantitative sample recovery, and therefore was employed as a means of purification for further experiments.

D. Influence of Several Parameters on Biological Properties and Radiolabeling Efficiency of Deferoxamine-Fibrinogen

Since the yield of ^{67}Ga-fibrinogen obtained in the experiments comparing dialysis with gel filtration was consistently low (less than 30%), altering parameters such as level of conjugation of deferoxamine with fibrinogen, and protein concentration were investigated to determine whether an increase in radiolabeling efficiency could be obtained with minimal impact on fibrinogen's biological activity. The analyses performed throughout this study are illustrated in Figure 10.

Several analyses such as the determination of protein concentration and percent clottability were performed repeatedly to evaluate whether any changes to the protein occurred during the reaction with deferoxamine-glutaraldehyde and also during purification of the protein complex. Deferoxamine concentration of the reaction product was determined along with each fraction collected during purification of deferoxamine-fibrinogen to ensure that all the deferoxamine was recovered from column chromatography. The measurement of deferoxamine concentration was also necessary to calculate the initial molar ratio (the ratio of deferoxamine to fibrinogen prior to purification of the reaction product), and final molar ratio (the level of conjugation of deferoxamine-fibrinogen after excess deferoxamine-glutaraldehyde was removed).

Upon completion of the radiolabeling procedure, paper chromatography involving two separate chromatography systems was utilized along with column chromatography to evaluate the yield of ^{67}Ga-fibrinogen and the nature of any free, non-protein bound radioactivity in the ^{67}Ga-fibrinogen solution. Paper chromatography was employed since Bio-Gel P-6DG alone could not separate ^{67}Ga-deferoxamine from ^{67}Ga-citrate. Paper chromatography, however, clearly separated ^{67}Ga-deferoxamine from ^{67}Ga-citrate when using the butanol:propanol:water solvent, while the pyridine:ethanol:water system separated ^{67}Ga-fibrinogen from the above compounds. SDS-PAGE and paper electrophoresis produced questionable results as to the nature of the compounds present and hence were not employed.

Isotopic clottability of the purified ^{67}Ga-fibrinogen fraction was included to study the relationship between protein clottability and the amount of radiogallium retained in the clot itself. Finally, the stability of the purified radiolabeled conjugate was measured by reapplying an aliquot of purified ^{67}Ga-fibrinogen to the Bio-Gel P-6DG column 6 hours and 24 hours after the radiolabeling procedure. Fractions were then collected and a u.v./radioactivity elution profile obtained in order to determine whether any activity was lost from the protein.

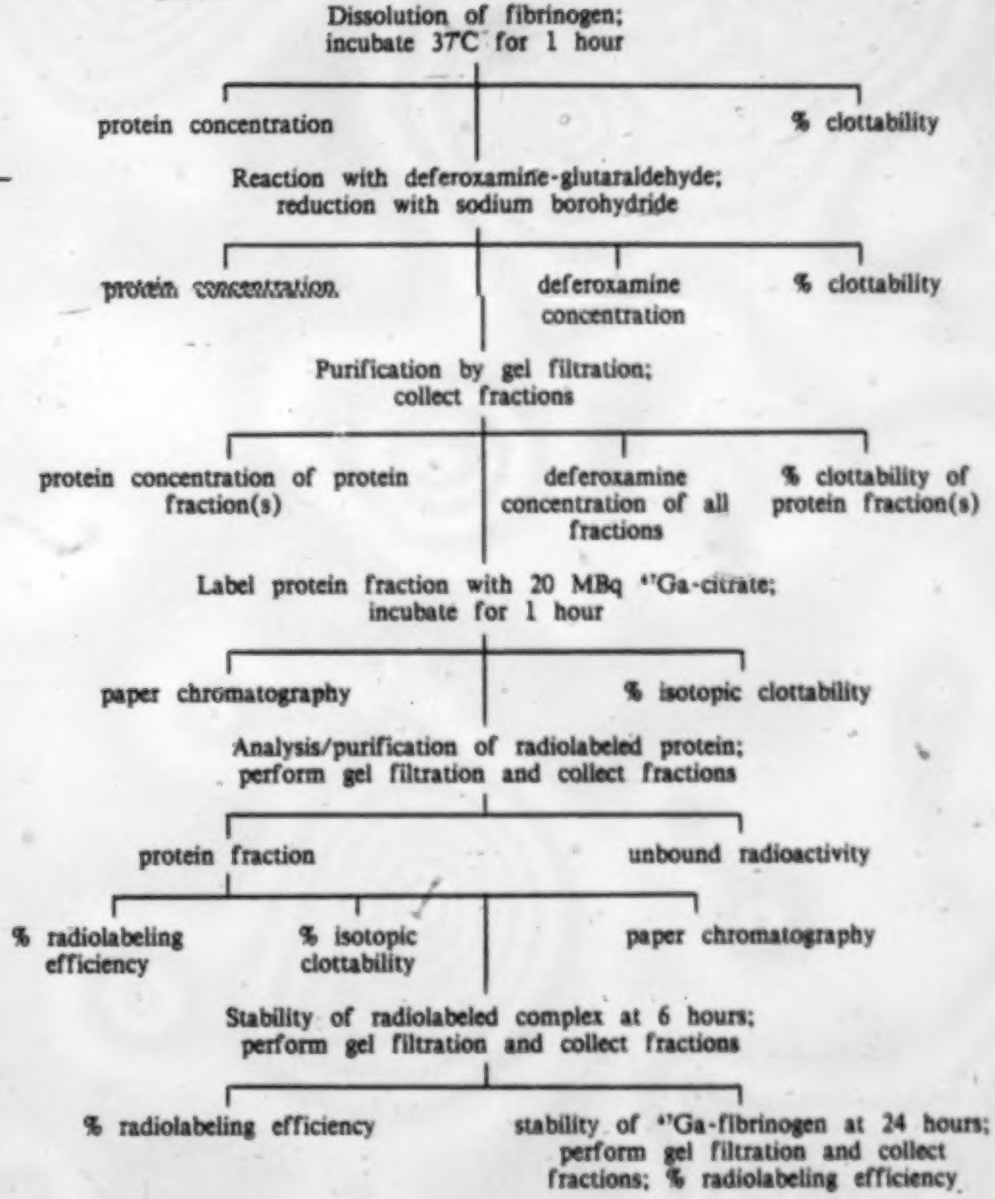

Dissolution of fibrinogen;
incubate 37°C for 1 hour
protein concentration
% clottability
Reaction with deferoxamine-glutaraldehyde;
reduction with sodium borohydride
protein concentration
deferoxamine concentration
% clottability
Purification by gel filtration;
collect fractions
protein concentration of protein fraction(s)
deferoxamine concentration of all fractions
% clottability of protein fraction(s)
Label protein fraction with 20 MBq ^{67}Ga-citrate;
incubate for 1 hour
paper chromatography
% isotopic clottability
Analysis/purification of radiolabeled protein;
perform gel filtration and collect fractions
protein fraction
unbound radioactivity
% radiolabeling efficiency
% isotopic clottability
paper chromatography
Stability of radiolabeled complex at 6 hours;
perform gel filtration and collect fractions
% radiolabeling efficiency
stability of ^{67}Ga-fibrinogen at 24 hours; perform gel filtration and collect fractions; % radiolabeling efficiency

Figure 10: Flow Chart Illustrating Analyses Employed Throughout the Preparation and Analysis of ^{67}Ga-Fibrinogen

1. Molar Ratio

It is known that the level of conjugation, i.e. the molar ratio of coupled chelating group to protein, affects both the biological properties and the specific activity of the radiolabeled protein (199). Although Ohmomo *et al* (77) utilized a molar ratio of three to six molecules of deferoxamine per molecule of fibrinogen, no data were reported to prove that the above ratio was actually the optimum conjugation level. Therefore, four different deferoxamine-fibrinogen products were prepared, these products varying only in the number of deferoxamine molecules linked to the protein via glutaraldehyde. (The deferoxamine-glutaraldehyde ratio was kept constant at 0.83 moles glutaraldehyde per mole deferoxamine.)

The initial concentrations of deferoxamine added to a 0.02 mM fibrinogen solution are given in Table 6. The deferoxamine concentration ranged from 191.0 nmoles/mL to 2,138.6 nmoles of deferoxamine per mL of reaction mixture. Once purified, the mean final molar ratios obtained ranged between 2.4 molecules of deferoxamine per molecule of fibrinogen (range=1.8:1 to 2.9:1, n=2) to 26.8:1 deferoxamine:fibrinogen (range=18.4:1 to 40.5:1, n=5).

The spectrophotometric assay developed by Emery and Hoffer (190) was used to determine the concentration of deferoxamine in the reaction mixture and in the purified product. The concentration of deferoxamine in solution could be quantitated with 95% confidence at levels greater than 26.4 nmoles/mL (the limit of detection). At concentrations between 13.2 and 26.4 nmoles/mL, deferoxamine could be detected but not quantitated with the same degree of confidence. When preparing deferoxamine-fibrinogen, employing a deferoxamine concentration of 191.0 nmoles/mL, the final concentration of the chelate, after purification of the protein complex, averaged 16.8 nmoles/mL (range = 14.3 to 19.4 nmoles/mL). It is realized that these values are not completely accurate; however, for the purpose of investigating the effects of a low deferoxamine-fibrinogen molar ratio on protein clottability and radiolabeling yield, these results were included in the study.

Table 6

Initial Deferoxamine Concentrations Employed During Coupling Reaction and Final Conjugation Levels Obtained

Initial Deferoxamine Concentration[1] (nmoles/mL)	Deferoxamine-Fibrinogen Molar Ratio Prior to Purification[2]	Final Deferoxamine Concentration* (nmoles/mL)[2]	Final Deferoxamine-Fibrinogen Molar Ratio[2]
191.0 (n = 2)	13.1:1 (12.4-13.8:1)	16.8 (14.3-19.4)	2.4:1 (1.8-2.9:1)
1,069.3 (n = 5)	61.6:1 (55.8-66.3:1)	53.8 (35.0-89.4)	7.1:1 (3.3-13.9:1)
1,568.7 (n = 2)	109.2:1 (100.7-117.8:1)	67.0 (66.3-67.6)	12.2:1 (12.0-12.4:1)
2,138.6 (n = 5)	125.3:1 (79.1-151.8:1)	171.6 (124.3-295.4)	26.8:1 (18.4-40.5:1)

[1] deferoxamine-glutaraldehyde ratio constant at 0.83 moles glutaraldehyde per mole deferoxamine

[2] values expressed as the mean and (range)

*Limit of Detection, L_D = 26.4 nmoles/mL;

*Critical Level of Detection, L_C = 13.2 nmoles/mL

Table 7

Effect of Deferoxamine Conjugation on Clottability of Fibrinogen

Initial Deferoxamine Concentration[1] (nmoles/mL)	Clottability of Deferoxamine-Fibrinogen Prior to Purification[2] %
191.0 (n = 2)	75.4* (73.2-77.7)
1,069.3 (n = 5)	69.0 (66.0-71.2)
1,568.7 (n = 2)	62.0 (56.9-67.0)
2,138.6 (n = 5)	58.3* (49.1-70.5)

[1] deferoxamine-glutaraldehyde ratio constant at 0.83 moles glutaraldehyde per mole deferoxamine

[2] values expressed as the mean percent clottability and (range)

*$P<0.05$

2. Effect of Conjugation on Percent Clottability of Fibrinogen

The incorporation of any foreign substance onto fibrinogen could seriously hamper its ability to form thrombi *in vivo*. Therefore, clottability is a sensitive *in vitro* indicator of fibrinogen lability.

In Table 7, the effect of conjugation levels on the clottability of fibrinogen is illustrated. When only 191.0 nmoles/mL of deferoxamine were added, the clottability of fibrinogen in the reaction mixture remained fairly high, with the clottability averaging 75.4% (range = 73.2% to 77.7% for n=2). As the amount of deferoxamine-glutaraldehyde increased, the clottability gradually decreased, and finally reached an average of 58.3% (range = 49.1% to 70.5%, n=5) when a maximum of 2,138.6 nmoles deferoxamine per mL was added. Although no statistical difference was observed between each consecutive level of deferoxamine-glutaraldehyde addition, a significant difference was seen between values obtained with the maximum amount of deferoxamine added and the minimum amount ($P<0.05$).

In Figure 11 a trend is also illustrated between the final molar ratio of deferoxamine-fibrinogen after purification with gel filtration, and the resultant clottability of deferoxamine-fibrinogen, the clottability gradually decreasing with increasing deferoxamine-fibrinogen molar ratios. If the line were extrapolated back to the y-axis, a clottability of 81.1% would be achieved, this corresponding to the clottability of native fibrinogen (average clottability of 79.8%, s.d.=1.5%, n=17).

Wensel and Meares (172) have suggested that if a specific conjugation takes place, one chelating equivalent per mole of protein is desirable. However if the conjugation is totally random, the desired extent of conjugation is approximately one chelating equivalent for every three to four moles of protein. The lower conjugation level for the random case is necessary due to the statistics of random conjugation (176). The statistics dictate that if an average of one mole of chelate is added per mole of protein, many of the protein molecules will actually contain more than one chelating group. These molecules are more likely to be inactivated or have different physical and chemical properties from their native counterparts.

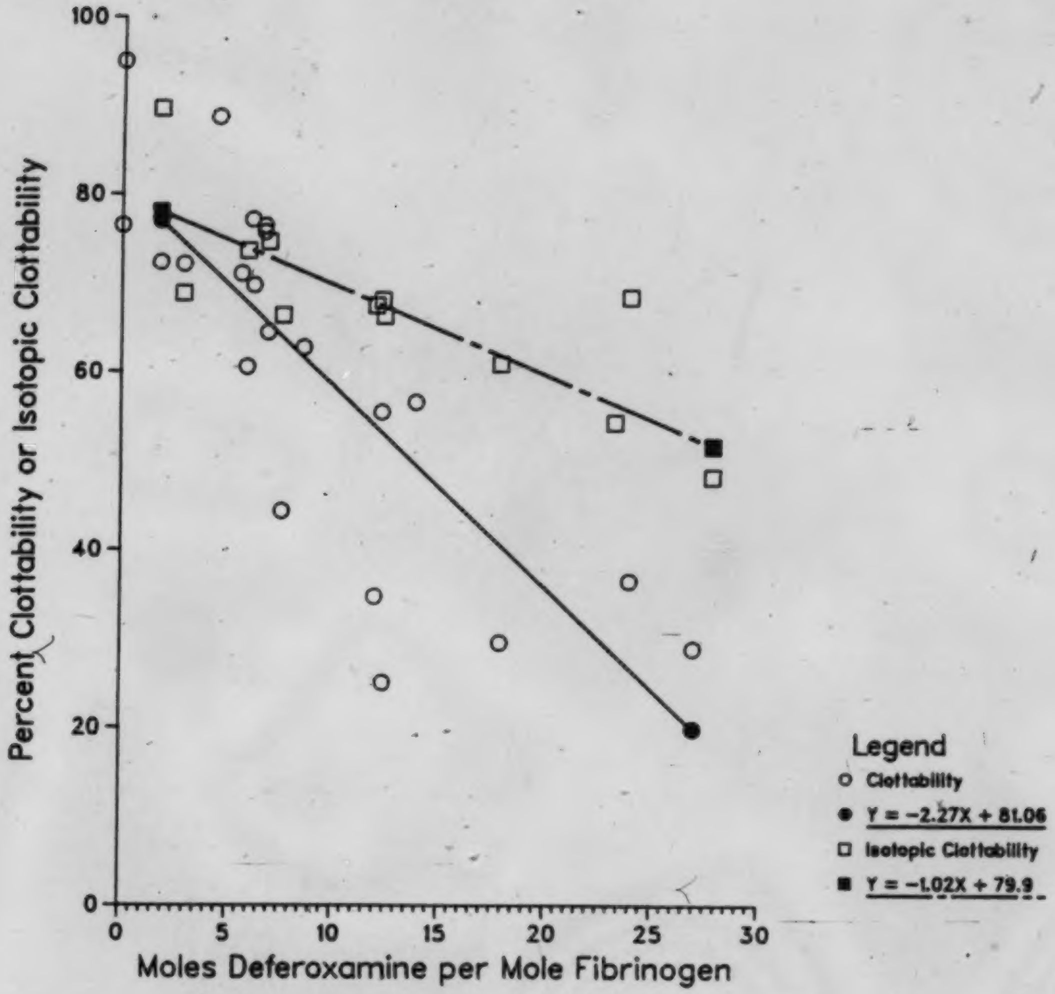


Figure 11: Effect of Level of Deferoxamine Conjugation on Clottability of Purified Deferoxamine-Fibrinogen, and Isotopic Clottability of Purified ^{67}Ga-Fibrinogen

Complexing fibrinogen with deferoxamine-glutaraldehyde can be considered as random conjugation. Glutaraldehyde will react extensively with α- and ε-amino groups, and sulphydryl groups (221), while the phenolic group of tyrosine and the imidazole ring of histidine tend to exhibit only moderate reactivity towards glutaraldehyde. However, since fibrinogen contains approximately 221 moles of lysine per mole of fibrinogen (123), and since fibrinogen has no free sulphydryl groups (124), the major reaction is with amino residues such as lysine. At higher deferoxamine-fibrinogen conjugation levels, it is probable that a portion of the fibrinogen molecules would be so heavily modified that their biological activity would be affected. In addition, these distorted molecules would tend to unfold, aggregate, and thus form a precipitate (241).

The appearance of increasing amounts of an insoluble gel-like material was observed during the reaction between deferoxamine-glutaraldehyde and fibrinogen, the rate and extent of insoluble material increasing with increasing molar ratios. In fact, when more than 2,138 nmoles/mL of deferoxamine was added, a very dense gel formed within 45 minutes. Thus, this indicates that some denaturation of the protein is occurring due to the reaction of deferoxamine-glutaraldehyde with fibrinogen.

The addition of sodium borohydride, necessary for reducing the imine bonds between the protein and the coupling agent glutaraldehyde, and between glutaraldehyde and deferoxamine, could also be implicated in the decrease in clottability and the precipitate formation observed. Sodium borohydride has the ability to cleave the disulfide bonds within fibrinogen, these bonds being essential for retaining the tertiary structure of fibrinogen. However, according to Means and Feeney (236), low concentrations of sodium borohydride should have no effect on disulfide bonds. These investigators studied the effect of reductive alkylation of amino groups, using formaldehyde and sodium borohydride, upon the enzymatic activity of ribonuclease A. They found that sodium borohydride alone (at a concentration of 0.5 - 1.0 mg/mL protein solution) had no effect on ribonuclease's enzymatic activity. Since the maximum amount of sodium borohydride added to fibrinogen during a reaction with deferoxamine-glutaraldehyde was less than 0.2 mg/mL, the decrease in clottability was not likely due to a reaction of the protein with sodium borohydride.

Another plausible explanation for the formation of a precipitate and a decrease in clottability may be due to the presence of free glutaraldehyde. As mentioned previously, glutaraldehyde was employed to couple deferoxamine with fibrinogen via a two-step procedure (219). The formation of deferoxamine-glutaraldehyde however is most probably not quantitative, i.e. four possible species may exist: free glutaraldehyde, free deferoxamine, deferoxamine-glutaraldehyde-deferoxamine and the desired deferoxamine-glutaraldehyde (see Figure 12). Thus, when an aliquot of deferoxamine-glutaraldehyde is added to fibrinogen, both glutaraldehyde and deferoxamine-glutaraldehyde will be competing for the lysine residues. Once glutaraldehyde is linked with one protein molecule, protein-protein coupling could easily occur, possibly forming a highly cross-linked fibrinogen polymer (237). This in turn could cause precipitation of the polymer. Since the ratio of glutaraldehyde to deferoxamine was kept constant, increasing the molar ratio of deferoxamine to fibrinogen actually increased the total amount of glutaraldehyde added to the protein. Therefore, when high molar ratios were employed, the large amount of insolubilized material observed may be due to protein-protein coupling.

The reaction of glutaraldehyde with deferoxamine and fibrinogen may actually be more complex than described above. Aqueous solutions of glutaraldehyde have been found to consist of free glutaraldehyde (1), the cyclic hemiacetal of its hydrate (2), and oligomers of this (3) in equilibrium with each other (Figure 13) (242). All of these samples react as free glutaraldehyde under acidic and neutral conditions of pH. Thus, the reaction of the reagent with the amino group of deferoxamine, and subsequently, with lysine moieties of proteins is complicated in nature, yielding a mixture of several products. It may, therefore, be these undesired products that cause the decrease in clottability and the polymerization of the protein.

The problem of protein polymerization has been observed by several investigators. Koizumi and coworkers (243) found that coupling reagents greatly affected the *in vitro* properties and *in vivo* distribution of a labeled monoclonal antibody to human chorionic

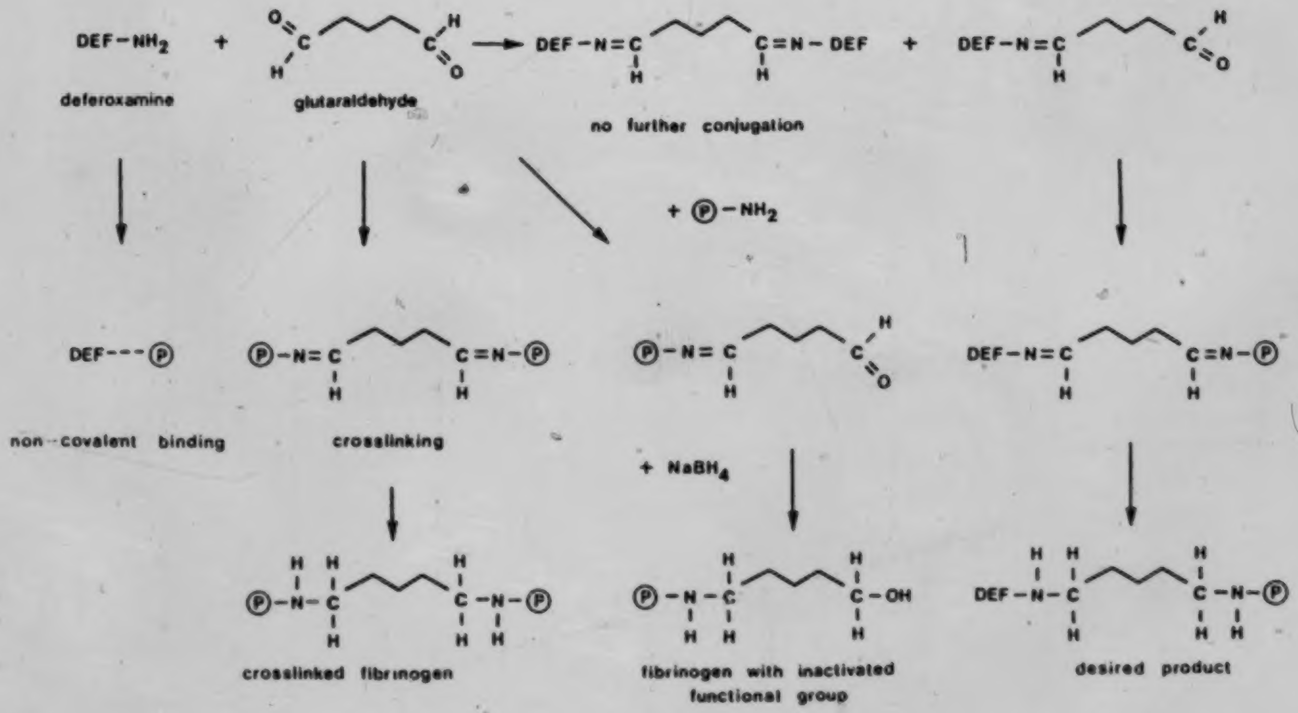
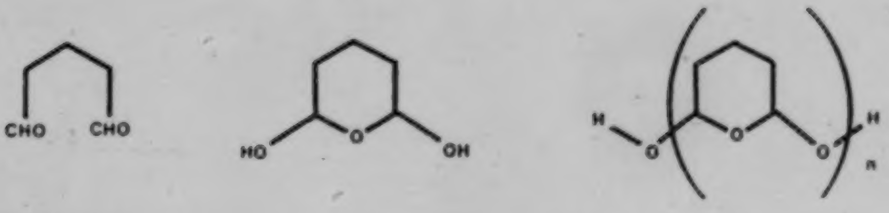

DEF−NH₂
deferoxamine
glutaraldehyde
no further conjugation
+ Ⓟ−NH₂
non-covalent binding
crosslinking
+ NaBH₄
crosslinked fibrinogen
fibrinogen with inactivated functional group
desired product

Figure 12: Postulation of Protein-Protein Coupling with Glutaraldehyde



(1) (2) (3)

Figure 13: Nature of the Reagent Glutaraldehyde in Aqueous Solution (242)

gonadotrophin, although the immunoreactivity, radiochemical purity and *in vitro* stability were satisfactory. Polymerization of the antibody was particularly a problem when glutaraldehyde was employed. Furthermore, Pritchard *et al* (223) found the formation of transferrin polymers when attempting to prepare IgG-transferrin using glutaraldehyde.

Certain investigators (214,237) have stated that glutaraldehyde reacts with amino groups not actively participating in the catalytic or immunological activity of proteins thus preserving the biological activity of individual proteins. Yet it is likely that a combination of factors, such as:

1. the presence of free glutaraldehyde in the protein solution,
2. the presence of impurities in the glutaraldehyde solution, and
3. the high deferoxamine-fibrinogen conjugation levels employed,

are causing a decrease in the clottability of the protein.

3. Effect of Conjugation on Isotopic Clottability After Purification of ^{67}Ga-Fibrinogen

For ^{67}Ga-fibrinogen to be an effective thrombus imaging agent *in vivo*, the amount of radiogallium retained in the clot should be sufficient to allow the thrombi to be easily visualized using a gamma camera. Thus, to determine whether a particular molar ratio yielded maximum incorporation of ^{67}Ga in a clot, or whether a trend between molar ratio and isotopic clottability existed, isotopic clottability experiments were performed with aliquots of purified ^{67}Ga-fibrinogen. The results of varying the deferoxamine-fibrinogen conjugation level on isotopic clottability are given in Table 8.

As the final molar ratio increased from 2.4 to 26.8 moles of deferoxamine per mole of fibrinogen, the mean isotopic clottability decreased from 79.2% to 61.5% respectively. Using the unpaired t-test, no statistical significance between the averaged results was observed. However, when individual final molar ratios were plotted against their respective isotopic clottabilities, a trend could be visualized (see Figure 11). As the molar ratio increased, the number of fibrinogen molecules which lost their ability to form a clot also increased. Yet these inactivated molecules are often still capable of binding gallium. Therefore when a sample of ^{67}Ga-fibrinogen was clotted, only those radiolabeled molecules which remained biologically active formed a clot leaving the heavily modified, radiolabeled macromolecules in the supernatant.

If ^{67}Ga distributed itself evenly between active and non-clottable deferoxamine-fibrinogen, one would expect to observe the lines in Figure 11 to run parallel with each other i.e. isotopic clottability should decrease at the same rate as protein clottability with increasing molar ratios. However, this was not the case, the isotopic clottability falling at a lower rate than protein clottability. Since the number of deferoxamine molecules far exceeds that number of gallium molecules added to deferoxamine-fibrinogen (20 MBq of ^{67}Ga being equivalent to 0.014 nmoles ^{67}Ga), gallium appears to preferentially bind with those deferoxamine molecules that are likely more accessible to the radiometal. Therefore if a fibrinogen molecule is heavily laden with deferoxamine, steric hindrance and/or a decrease in deferoxamine metal binding capacity may prevent ^{67}Ga from chelating with denatured deferoxamine-fibrinogen (241,244). Thus, preferential binding of ^{67}Ga with the clottable protein complex would be observed.

4. Effect of Conjugation on Radiolabeling Efficiency

In contrast with the technique used for determining isotopic clottability, gel filtration does not differentiate between clottable and non-clottable radiolabeled protein. However, evaluating the yield of ^{67}Ga-fibrinogen is an important step in determining its specific activity

Table 8

Effect of Deferoxamine Conjugation on Isotopic Clottability[1]

Final Molar Ratio[2]	Isotopic Clottability[•] (%)
2.4:1 (n = 2)	79.2 (68.8-89.7)
7.1:1 (n = 5)	70.2 (55.2-77.4)
12.2:1 (n = 2)	66.8 (66.2-67.4)
26.8:1 (n = 5)	61.5 (48.1-72.3)

[1] values expressed as the mean percent isotopic clottability, after purification of ^{67}Ga-fibrinogen by gel filtration, and (range)

[2] moles of deferoxamine per mole of fibrinogen after purification of the protein complex

[•] no statistically significant difference observed (unpaired t-test)

Table 9

Effect of Deferoxamine Conjugation on Radiolabeling Efficiency

Final Molar Ratio[1]	Percent Labeling Efficiency[2]	Percent[2] of Total Radioactivity[3] as Unbound ^{67}Ga-Deferoxamine[4]
2.4:1 (n = 2)	2.5 (1.5-3.5)	N.D.[•]
7.1:1 (n = 5)	42.3 (27.2-63.4)	1.6 (0.0-5.0)
12.2:1 (n = 2)	34.6 (33.7-35.4)	13.2 (12.1-14.4)
26.8 (n = 5)	22.3 (2.8-45.0)	44.4 (25.3-69.6)

[1] moles of deferoxamine per mole of fibrinogen after purification of the protein complex

[2] values expressed as the mean percent and (range)

[3] addition of 20 MBq of ^{67}Ga-citrate

[4] as determined by paper chromatography; remainder of non-protein bound radioactivity as ^{67}Ga-citrate

[•] none detected

i.e. the amount of radioactivity bound per unit weight of protein. As mentioned previously, it is desirable to maximize the specific activity of ^{67}Ga-fibrinogen. Theoretically, one approach may be to increase the level of conjugation of fibrinogen with deferoxamine. By increasing the molar ratio, the amount of deferoxamine linked to fibrinogen and available for chelating ^{67}Ga should increase, resulting in a higher yield of ^{67}Ga-fibrinogen. However according to the results in Table 9, radiolabeling efficiency actually decreased at higher molar ratios. At a molar ratio of 2.4:1 deferoxamine:protein, only 2.5% of ^{67}Ga was complexed to the protein. At a deferoxamine-fibrinogen conjugation level of 7.1:1, the yield of ^{67}Ga-fibrinogen increased to 42.3%. However, further increases in molar ratio resulted in the mean radiolabeling efficiency decreasing to 34.6% and 22.3% for deferoxamine-fibrinogen ratios of 12.2:1 and 26.8:1 respectively.

As shown in Figure 14, it appears that the separation of deferoxamine-fibrinogen from unreacted deferoxamine on column chromatography was adequate. However, paper chromatography of aliquots of ^{67}Ga-fibrinogen, prior to purification, revealed increasing amounts of non-protein bound ^{67}Ga-deferoxamine in the ^{67}Ga-fibrinogen solution. Up to 44.4% of the total radioactivity was actually bound to unreacted deferoxamine at high molar ratios with only 22.3% chelated with deferoxamine-fibrinogen. The remaining radioactivity was identified as ^{67}Ga-citrate. These results would indicate that:

1. separation using the 40 mL column was incomplete, and/or
2. non-covalently bound ^{67}Ga-deferoxamine was slowly being released from fibrinogen (171).

If non-covalently bound deferoxamine was slowly being leached from the protein complex, the appearance of ^{67}Ga-deferoxamine should also have been observed at low molar ratios. However at a conjugation level of 2.4 moles of deferoxamine per mole of fibrinogen, no free ^{67}Ga-deferoxamine was detected with paper chromatography. Likewise, only 1.6% of the total radioactivity existed as non-protein bound ^{67}Ga-deferoxamine when a molar ratio of 7.1:1 was employed. Therefore, to determine whether incomplete separation of deferoxamine-fibrinogen from free deferoxamine was the reason for the radiolabeling efficiencies observed, a

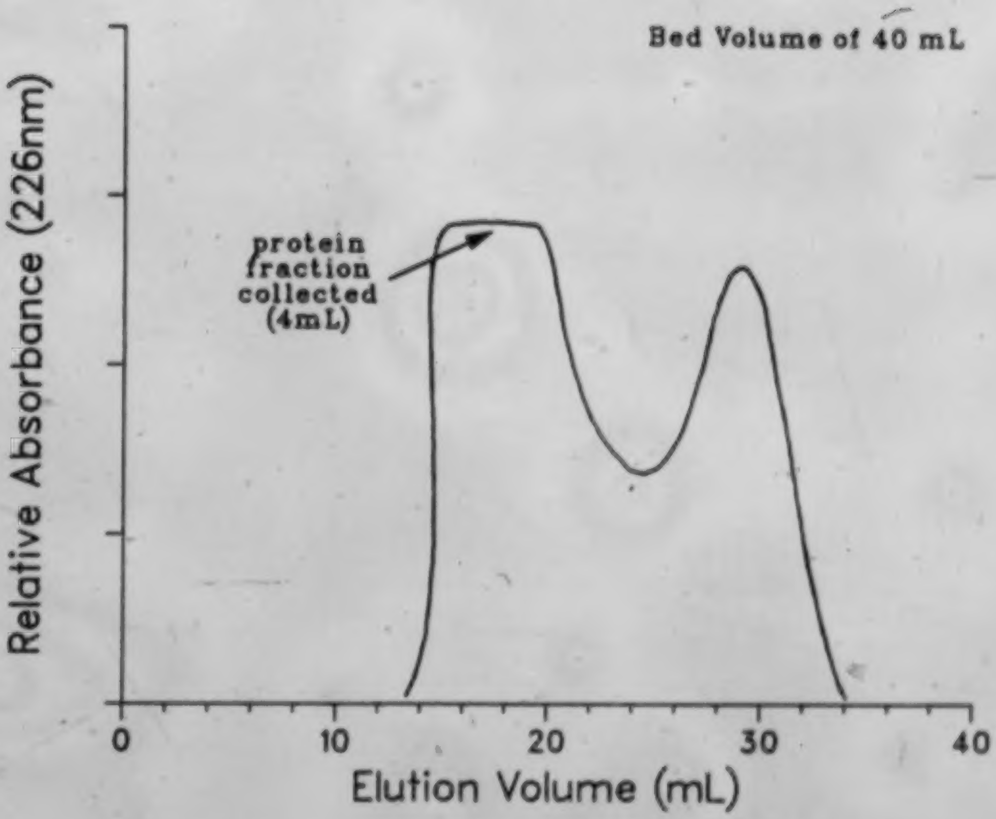


Figure 14: Purification of Deferoxamine-Fibrinogen by Bio-Gel P-6DG Gel Filtration

longer Bio-Gel P-6DG column was prepared for purification of deferoxamine-fibrinogen.

5. Effect of Increasing Column Length for Purification of Deferoxamine-Fibrinogen

Increasing the column length increases the HETP (height equivalent theoretical plate) value and thus increases resolution (245). However sample dilution will also increase as will transit time of the sample through the column. The results of lengthening the Bio-Gel P-6DG column from 40 mL to 65 mL are given in Table 10.

Three different concentrations of deferoxamine were added to fibrinogen: 191.0 nmoles/mL, 1,069.3 nmoles/mL, and 2,138.6 nmoles of deferoxamine per mL of fibrinogen solution. A 2.0%, 33.2% and 60.1% radiolabeling efficiency, corresponding with the above deferoxamine concentrations, was observed. These results differ from those values obtained when employing a 40 mL column, where at 1,069.3 and 2,138.6 nmoles of deferoxamine per mL, the yield of ^{67}Ga-fibrinogen attained was 42.3% and 22.3% respectively (Table 9). In addition, the amount of free radioactivity occurring as ^{67}Ga-deferoxamine decreased from 44.4% (with the short column) to only 3.1% (65 mL column) at high deferoxamine concentrations. Non-protein bound ^{67}Ga-deferoxamine was not detected when lower concentrations of deferoxamine were used. Therefore it is evident that the 40 mL Bio-Gel column was not adequate in effectively separating the unbound chelating agent from the protein-chelate complex when employing high amounts of deferoxamine, thereby decreasing the observed percent yield of ^{67}Ga-fibrinogen. Yet it still remained difficult to completely separate unreacted deferoxamine from deferoxamine-fibrinogen at high deferoxamine concentrations even when a longer gel filtration column was used. It is interesting to note that Layne *et al* (42) had difficulty purifying DTPA-fibrinogen, and found it necessary to perform two purification steps in order to remove the excess DTPA from the protein complex.

The final deferoxamine-fibrinogen molar ratios obtained with the two Bio-Gel columns varied considerably when the same initial concentrations of deferoxamine were reacted with fibrinogen. For instance, at an initial deferoxamine concentration of 2,138.6 nmoles/mL, a final molar ratio obtained with a 65 mL column averaged 7.2 moles

Table 10

Results of Various Parameters After Increasing the Column Length for Purification of Deferoxamine-Fibrinogen[1]

Initial Deferoxamine Concentration[2] (nmoles/mL)	Final Molar Ratio[3]	Percent Labeling Efficiency	Percent of Total Radioactivity as ^{67}Ga-Deferoxamine[4]	Percent Clottability[5]	Percent Isotopic Clottability[6]
191.0	2.6:1 (2.0-3.3:1)	2.0 (0.1-4.0)	N.D.[*]	67.4 (64.3-70.4)	72.3 (72.0-72.6)
1,069.3	4.2:1 (3.6-4.8:1)	33.2 (25.3-41.2)	N.D.[*]	52.0 (46.2-57.7)	72.8 (70.8-74.7)
2,138.6	7.2:1 (7.1-7.2:1)	60.1 (52.5-67.7)	3.1 (2.6-3.6)	48.2 (40.6-55.7)	71.1 (67.8-74.4)

[1] mean and (range) of duplicate determinations

[2] deferoxamine-glutaraldehyde ratio constant at 0.83 moles glutaraldehyde per mole deferoxamine

[3] moles of deferoxamine per mole of fibrinogen after purification of the protein complex

[4] as determined by paper chromatography; remaining non-protein bound radioactivity as ^{67}Ga-citrate

[5] after purification of deferoxamine-fibrinogen by column chromatography

[6] after purification of ^{67}Ga-fibrinogen by column chromatography

[*] none detected

deferoxamine per mole fibrinogen versus a final molar ratio of 26.8:1 with a 40 mL column. Likewise, a 7.1:1 versus a 4.2:1 deferoxamine-fibrinogen molar ratio was attained with a 40 mL and 65 mL column respectively when the initial deferoxamine concentration was 1,069.3 nmoles/mL. At low deferoxamine concentrations (191.0 nmoles/mL) very little difference between the final molar ratios was discernible (2.4:1 and 2.6:1 for the shorter and longer columns).

The above results would suggest that when the 40 mL gel filtration column was utilized, unbound deferoxamine was not completely removed from the deferoxamine-protein complex (at initial deferoxamine concentrations greater than 191.0 nmoles/mL). However, if this was the case, at an intermediate molar ratio of 7.1:1 (40 mL column), a larger percentage of non-protein bound radioactivity in the ^{67}Ga-fibrinogen product should be present as ^{67}Ga-deferoxamine. Yet out of five experiments performed, producing an average conjugation level of 7.1:1, only once was free ^{67}Ga-deferoxamine observed by paper chromatography, and its presence constituted only 5% of the total radioactivity. Furthermore, when comparing the radiolabeling efficiencies between similarly prepared ^{67}Ga-fibrinogen products purified by two different columns, it was found that the radiolabeling efficiency was higher for the product purified by the shorter column (molar ratio = 7.1:1, efficiency = 42.3% v.s. molar ratio 4.2:1, efficiency of 33.2%). If this was due to weak non-specific binding of deferoxamine to fibrinogen then the non-covalently bound chelate would be revealed as ^{67}Ga-deferoxamine in the 24 hour stability studies performed. However, no free radioactivity was detected after 24 hours. Therefore a small fraction of deferoxamine must be very tightly adsorbed to fibrinogen.

This is consistent with the work of Janoki *et al* (171) who found that 75-97% of free deferoxamine could be removed from deferoxamine-HSA by extensive purification (i.e. dialysis for 48 hours, gel filtration, then dialysis again with hollow fibre bundles). Still, 1-3% of the deferoxamine remained tightly adsorbed to the protein and could only be removed following urea treatment. (Wagner and Welch (54) had also observed similar problems in their attempts to purify an HSA-DTPA complex.) Janoki also observed that the stability of the radiolabeled conjugate remained greater than 90% after one week storage at 4°C. The radioactivity lost from the protein was all present as ^{67}Ga-deferoxamine which, the author speculated, may have corresponded with a very slow release of non-covalently bound deferoxamine from the protein complex.

The stability studies performed on all purified ^{67}Ga-fibrinogen products with varying molar ratios agree with Janoki's studies and indicate that no free radioactivity was lost from the protein complex. The u.v./radioactivity elution profiles of purified ^{67}Ga-fibrinogen, reapplied to a Bio-Gel column 6 hours and 24 hours after deferoxamine-fibrinogen preparation, consistently showed all the radioactivity remaining with the protein (see Figure 15).

There was no statistical difference between the products obtained from purification by the two columns with respect to percent clottability, isotopic clottability, and radiolabeling efficiency of deferoxamine-fibrinogen, when low to intermediate concentrations of deferoxamine were employed. In addition, the protein concentration of the eluted product was only marginally lower when a 65 mL column was used. However there are two main disadvantages in utilizing a longer column:

1. the time required for purification is doubled: 96 minutes v.s. 45 minutes for the 40 mL column, and
2. polymerization is observed during the purification process.

When the column bed volume was increased from 40 mL to 65 mL, a viscous solution was observed as the deferoxamine-fibrinogen complex was eluted. This gel formation was evident in all deferoxamine-fibrinogen preparations purified with a longer column, but was more pronounced when high concentrations of deferoxamine-glutaraldehyde were reacted with fibrinogen. Occasionally the viscosity was so great as to block the flow cell in the u.v. monitor. The formation of a gel when employing a 40 mL column for purification was only evident when the fibrinogen solution was allowed to incubate for 3 to 4 hours to allow complete dissolution of the protein prior to the reaction with deferoxamine-glutaraldehyde. Thus it appears that the rate of polymerization of deferoxamine-fibrinogen is enhanced by an

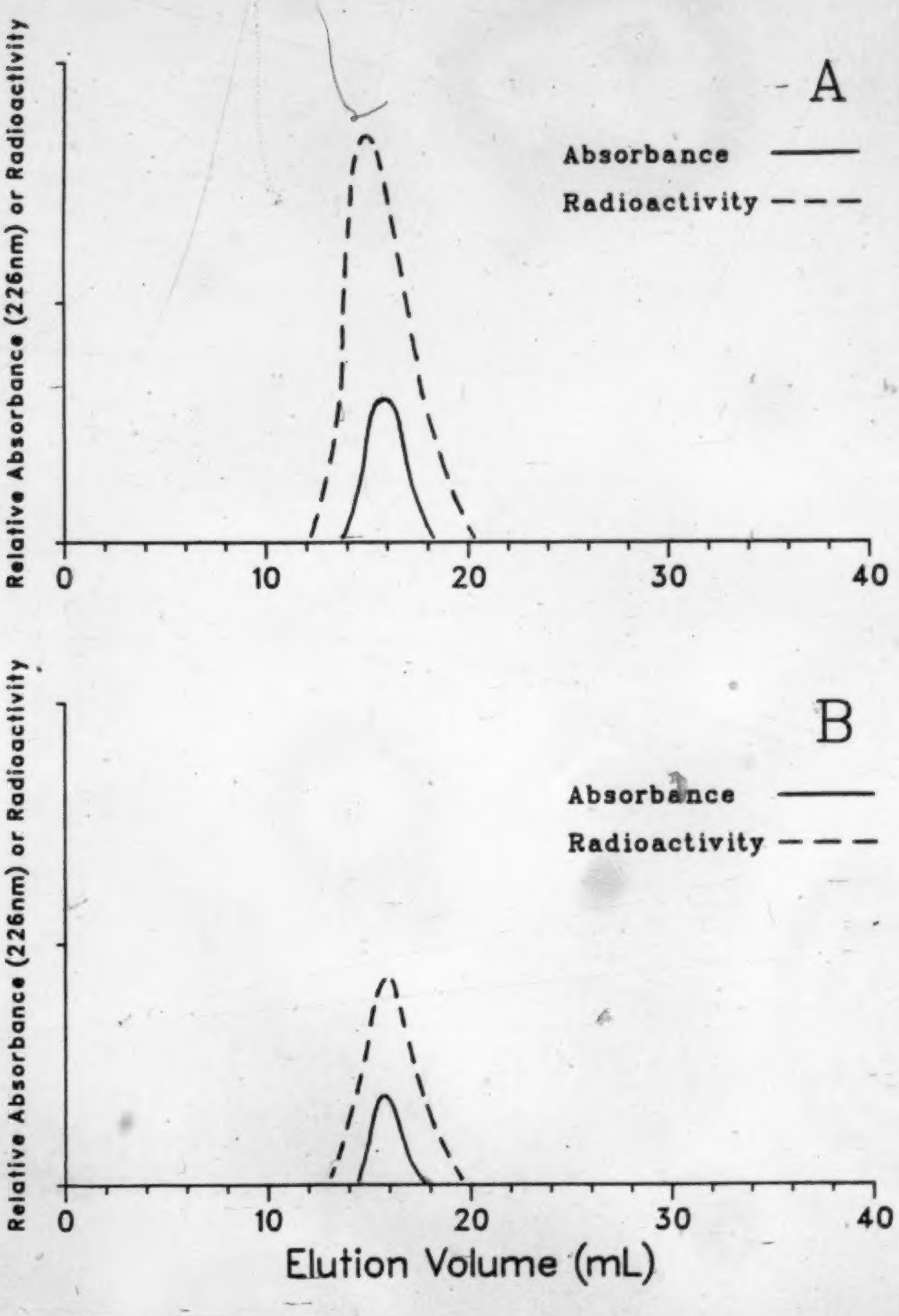


Figure 15: Stability Studies of ^{67}Ga-Fibrinogen at (A) 6 hours and (B) 24 hours by Bio-Gel P-6DG Column Chromatography

increased transit time during column chromatography.

In summary, the least amount of manipulation of fibrinogen possible is necessary in order for the protein to retain its clottability; yet, increasing the number of chelating molecules linked to a protein molecule results in a desirable increase in radiolabeling efficiency. Consequently, a conjugation level of approximately 7.1 moles of deferoxamine per mole of fibrinogen was chosen as the optimum molar ratio for preparing the deferoxamine-fibrinogen complex (using a 40 mL Bio-Gel P-6DG column for purification of deferoxamine-fibrinogen). Although great care was taken to obtain reproducible deferoxamine-fibrinogen molar ratios, the final conjugation levels attained varied considerably. Thus, individual deferoxamine-fibrinogen conjugation levels obtained experimentally were 6.9, 5.9, 13.9, 5.6, and 3.3:1. Except for one unusually high molar ratio, the remaining conjugation levels generally fell within the range quoted by Ohmomo *et al* (77) (conjugation level of 3 to 6:1 deferoxamine:fibrinogen).

6. Effect of Increasing Protein Concentration

Having determined an optimal molar ratio for binding deferoxamine with fibrinogen, an attempt to increase the radiolabeling efficiency was made by increasing the protein concentration from 6.8 mg/mL to 10.2 mg/mL. Increasing the protein concentration while maintaining the molar ratio should, in theory, increase the total number of chelate molecules available for complexation, resulting in an increase in radiolabeling efficiency.

As depicted in Table 11, the radiolabeling efficiency did not improve by increasing the protein concentration. Rather, the yield of ^{67}Ga-fibrinogen decreased dramatically to 7.7% from 42.3% ($P<0.01$) when 10.2 mg of fibrinogen per mL was employed. Since all the non-protein bound radioactivity behaved as ^{67}Ga-citrate rather than ^{67}Ga-deferoxamine, incomplete separation of deferoxamine-fibrinogen from unreacted deferoxamine does not explain the reason for a decrease in radiolabeling efficiency. Both Janoki *et al* (171) and Leung *et al* (189) proposed that chelating molecules became 'buried' within a protein complex due to inter- and intramolecular crosslinking. These molecules would not be available to bind

Table 11

Effect of Increasing Protein Concentration on Clottability[1], Isotopic Clottability[2], and Yield of ^{67}Ga-Fibrinogen

Initial Protein Concentration (mg/mL)	Final Protein Concentration after Purification[3] (mg/mL)	Final Molar Ratio[3,4]	Clottability[3] (%)	Isotopic Clottability[3] (%)	Radiolabeling Efficiency[3] (%)
6.8 (n = 5)	2.75* (2.11-3.60)	7.1:1 (3.3-13.9:1)	62.6* (56.5-71.0)	70.2 (55.2-77.4)	42.3** (27.2-63.4)
10.2 (n = 3)	1.82* (1.63-2.11)	11.3:1 (3.7-17.9:1)	43.0* (29.5-55.4)	58.9 (47.8-60.8)	7.7** (2.8-12.8)

[1] after purification of deferoxamine-fibrinogen by column chromatography

[2] after purification of ^{67}Ga-fibrinogen by column chromatography

[3] values expressed as the mean and (range)

[4] moles of deferoxamine per mole of fibrinogen after purification of the protein complex

*$P<0.05$

**$P<0.01$

radiometals and thus a low radiolabeling efficiency would result. When increasing the protein concentration to 10.2 mg/mL, an increased amount of free glutaraldehyde was also added (since the initial deferoxamine-fibrinogen molar ratio of 61.6:1 was maintained). The rate and extent of protein-protein coupling would then likely increase. This might cause deferoxamine to become trapped within the polymer,thus preventing the chelating agent from binding with ^{67}Ga, and causing a decrease in ^{67}Ga-fibrinogen yield.

Glazer (244) has suggested that functional groups are not necessarily 'buried' within a molecule; rather, functional groups on the exterior of a macromolecule are often sterically hindered. With an increase in protein concentration, the close vicinity of fibrinogen molecules may prevent the coupled deferoxamine molecules from binding radiogallium.

With respect to the final protein concentration, a significant decrease in protein concentration was observed after purification (1.82 v.s. 2.75 mg/mL, $P<0.05$), although a more concentrated fibrinogen solution was originally used. This decrease in final protein concentration was unexpected since an increase would be anticipated when purifying a more concentrated solution. However, during the reaction with deferoxamine-fibrinogen and also during purification, varying amounts of insolublized material were observed. The precipitation of fibrinogen would, in turn, decrease the protein concentration.

Clottability of fibrinogen was also significantly affected, the percent clottability decreasing to 43.0% (range = 29.5% to 55.4%) from 62.6% (range = 56.5% to 71.0%) ($P<0.05$). Due to higher concentrations of protein and free glutaraldehyde in solution, the increased risk of protein-protein coupling could result in polymerization and a decrease in protein clottability.

Isotopic clottability was also found to decrease from 70.2% (6.8 mg/mL fibrinogen) to 58.9% (10.2 mg/mL fibrinogen) although the difference was not statistically significant at the $P<0.05$ level. With a molar ratio of 11.3:1, many fibrinogen molecules likely carried a greater number of deferoxamine molecules than the average number calculated, due to random conjugation. Even if these modified protein molecules retained their ability to clot, the possibility of steric hindrance may have prevented the attached deferoxamine molecules from binding ^{67}Ga; thus, a decrease in isotopic clottability would be observed.

Although increasing the protein concentration while maintaining an initial molar ratio of 61.6:1 should, in theory, increase radiolabeling efficiency, the results indicated that this in fact adversely affected not only the yield of ^{67}Ga-fibrinogen but also the isotopic clottability, protein clottability, and the final protein concentration as well.

E. Preparation of High Specific Activity ^{67}Ga-Fibrinogen

^{67}Ga-fibrinogen has the potential to become a useful thrombus-imaging agent if its specific activity (the amount of radiogallium linked per unit weight of protein) could be increased. In theory, it would be desirable to label 1 to 2 mg of fibrinogen with 40 to 160 MBq of ^{67}Ga for clinical use. To determine whether the derivatized protein could accommodate larger quantities of radioactivity, the amount of radioactivity added to 1.0 mL of deferoxamine-fibrinogen (2 to 3 mg of protein) was increased from 20 MBq to 40 MBq of ^{67}Ga-citrate. The results are shown in Table 12.

Although the radiolabeling efficiency was greater when 20 MBq of radioactivity was added to deferoxamine-fibrinogen than when 40 MBq ^{67}Ga-citrate was added (42.3% and 12.2% respectively), no statistical difference between the radiolabeling efficiencies nor the specific activities was observed (2.08 MBq/mg with 40 MBq radioactivity versus 3.02 MBq/mg with 20 MBq ^{67}Ga-citrate). From these results, it appears that under the reaction conditions used, there is a limit to the amount of ^{67}Ga that may be linked with deferoxamine-fibrinogen, this limit ranging from 2 to 3 MBq per mg of protein.

At a molar ratio in the range of 7.1:1 deferoxamine:protein, 1 mg of protein complex should theoretically be able to chelate approximately 31 GBq of ^{67}Ga from ^{67}Ga-citrate (10,000-15,000 times more than was found experimentally). Incomplete transfer of ^{67}Ga from its citrate complex to deferoxamine could be one possible explanation for the low specific activity obtained. However, Weiner *et al* (57) found that ^{67}Ga-deferoxamine was rapidly and almost completely formed within 15 minutes (at room temperature and regardless of pH employed). Since deferoxamine-fibrinogen was allowed to incubate with ^{67}Ga-citrate for 1

Table 12

Effect of Increasing the Amount of Radioactivity Added to Deferoxamine-Fibrinogen on the Yield of ^{67}Ga-Fibrinogen

Amount of ^{67}Ga-Citrate Added to 1.0 mL Deferoxamine-Fibrinogen (MBq)	Protein Concentration[1] (mg/mL)	Radiolabeling Efficiency[1] (%)	Specific Activity[1] (MBq/mg protein)
20 (n = 5)	2.75 (2.11-3.60)	42.3* (27.2-63.4)	3.02* (1.72-3.52)
40 (n = 2)	2.29 (2.11-2.48)	12.2* (8.4-15.9)	2.08* (1.59-2.56)

[1] values expressed as the mean and (range)

*no statistically significant difference observed (unpaired t-test) hour, incomplete exchange of ^{67}Ga between citrate and deferoxamine was not likely the cause of the low radiolabeling efficiency obtained.

Janoki's (171) and Leung's (189) proposal of the deferoxamine molecule becoming buried within a polymeric complex was also not likely the sole reason for the low specific activity observed. Since deferoxamine concentration and molar ratio were both determined by complexing iron with an aliquot of deferoxamine-fibrinogen solution, and since the formation constant is supposedly higher for ^{67}Ga-deferoxamine than for ferrioxamine (60), there was no reason to believe that this same number of deferoxamine molecules was not capable of chelating gallium. However, if other metal ions were present in the ^{67}Ga-citrate solution, these ions may have competed with radiogallium for deferoxamine. Therefore an experiment was performed to determine whether metal contamination could be the reason for the low radiolabeling efficiencies (and low specific activities) observed.

F. Determination of Metal Contamination of ^{67}Ga-Citrate

Hnatowich *et al* (183) have suggested that trace metal contamination of $^{67}GaCl_3$ and ^{67}Ga-citrate solutions hinder the formation of ^{67}Ga-DTPA complexes even when high concentrations of DTPA are used. Turner *et al* (227) have shown that iron and zinc metals were the major contaminants in $^{67}GaCl_3$ and $^{111}InCl_3$ thus hampering the chelation of ^{67}Ga and ^{111}In to an IgG-EDTA complex. In fact, only 13.3% of the $^{67}GaCl_3$ could be incorporated when a large amount of chelate (663 nmoles EDTA) was complexed with IgG. Although the formation constant of ^{67}Ga-deferoxamine is much higher than that of Zn-deferoxamine (log $K>31$ versus log $K=10.1$ respectively) (59,60), large amounts of zinc metal present in ^{67}Ga-citrate could effectively compete with gallium for deferoxamine. Likewise iron, with a stability constant of log $K=30.6$ (59), would also vie with radiogallium for complexation with deferoxamine. Therefore, neutron activation analysis (NAA) was employed to ascertain whether significant amounts of zinc and/or iron were contained in ^{67}Ga-citrate solutions.

Using NAA, zinc and iron metal will form radioactive ^{65}Zn and ^{59}Fe respectively. The major γ-ray energies produced after a 4 hour irradiation at a flux of 1×10^{12} n·cm^{-2}s^{-1} and a cool time of 3 weeks are (159):

1. ^{65}Zn - 511 keV; abundance ≈3%,
2. ^{65}Zn - 1116 keV; abundance 51%,
3. ^{59}Fe - 1099 keV; abundance 56%, and
4. ^{59}Fe - 1292 keV; abundance 44%.

Comparing an irradiated sample of ^{67}Ga-citrate (Figure 16B), with a standard containing both radioactive zinc and iron (Figure 16A), two photopeaks are evident; that is, a 511 keV peak and a 1116 keV peak are present, indicating that zinc exists in the ^{67}Ga-citrate solutions. The photopeaks of ^{59}Fe were not detected in any of the ^{67}Ga-citrate samples analyzed.

As shown in Table 13, the amount of zinc in ^{67}Ga-citrate ranged from 18.9 to 3,110 nmoles/mL. Considering that the average concentration of deferoxamine is 53.8 nmoles/mL in the purified protein complex, and that 20 MBq of ^{67}Ga-citrate, equivalent to only 0.014 nmoles of ^{67}Ga, contained 5.1 to 764.0 nmoles of zinc (on the day of calibration), the possibility of competition between ^{67}Ga and zinc for chelation with deferoxamine does exist. Depending on the day of the week radiolabeling of deferoxamine-fibrinogen was performed, the amount of zinc added would vary, i.e. the volume containing 20 MBq ^{67}Ga-citrate would change due to decay of ^{67}Ga. Therefore, quantitative radiolabeling of deferoxamine-fibrinogen was possibly inhibited by the presence of zinc metal in the ^{67}Ga-citrate solution. Since the amount of zinc varied from batch to batch of ^{67}Ga-citrate, metal contamination could also account for the variability in radiolabeling efficiencies observed.

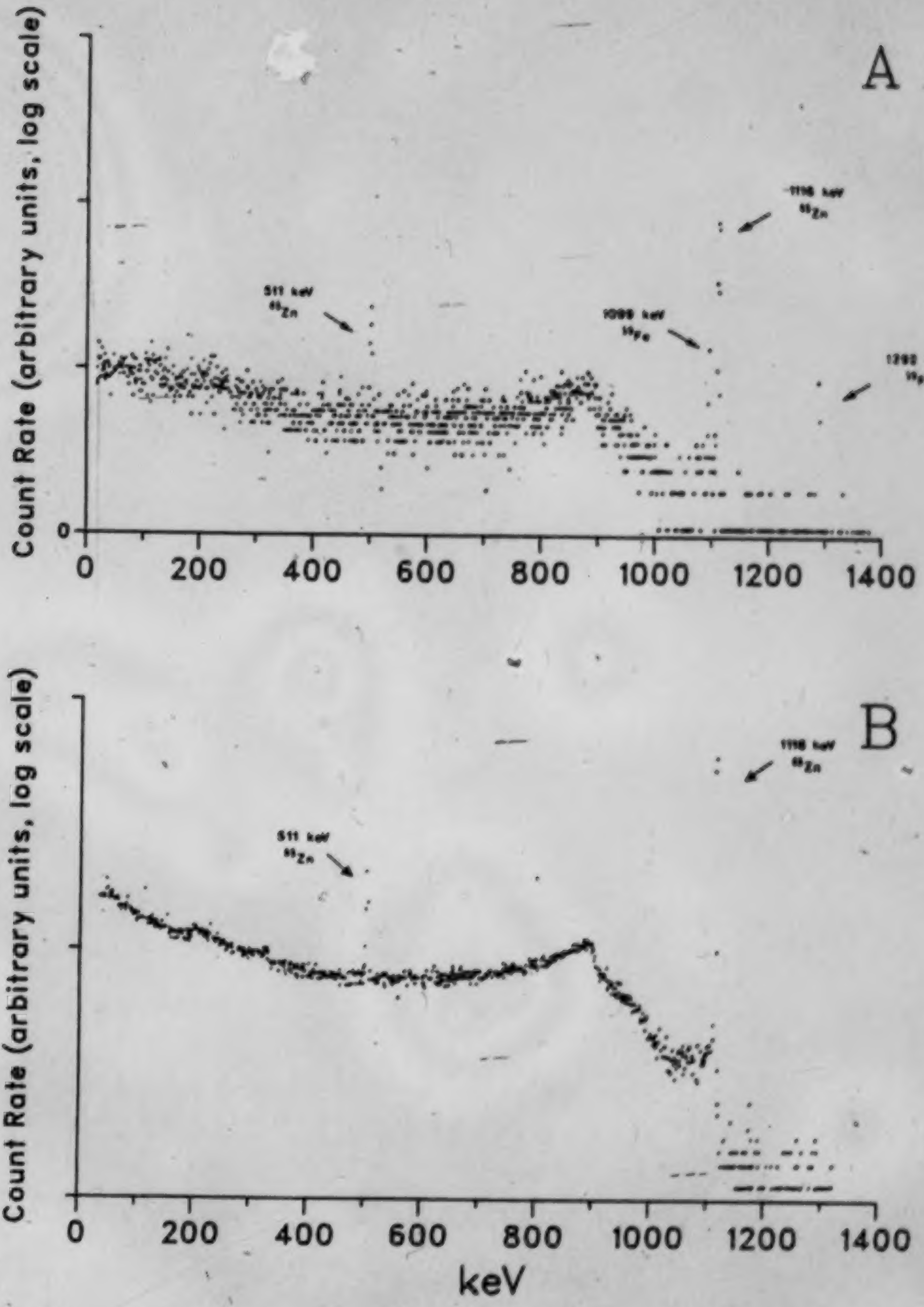


Figure 16: GeLi Spectra of (A) 100 μg Zinc and Iron Standard and (B) a ^{67}Ga-Citrate Sample, 3 Weeks after Thermal Irradiation for 4 hours at 10^{13} n·cm^{-2}s^{-1}

Table 13

Zinc Metal Contamination in ^{67}Ga-Citrate Samples

Sample	Zinc Concentration (nmoles/mL)	Percent Error (1σ)	L_D[1] (nmoles/mL)	Amount of Zinc Present in 20 MBq ^{67}Ga-Citrate[2] (nmoles)
A Lot 79220-1	236	9.2	6.9	57.9
B Lot 79266-1	137	9.2	4.2	33.8
C Lot BGa126	18.9	17.8	3.4	5.1
D Lot 80772-5	3,110	8.7	9.4	764.0

[1] Limit of Detection

[2] on day of calibration

V. SUMMARY AND CONCLUSIONS

1. ^{67}Ga-fibrinogen was prepared, utilizing a three-step procedure consisting of (i) coupling the chelating agent, deferoxamine, to fibrinogen via glutaraldehyde, (ii) purifying the protein complex, and (iii) subsequently radiolabeling the deferoxamine-fibrinogen complex with ^{67}Ga.
2. The effects of several buffer systems and radiolabeling agents on the formation of ^{67}Ga-deferoxamine were investigated. Acetate buffered saline, 0.1M, pH 7.4, was found to be the best medium when compared to phosphate buffered saline or citrate buffered saline. Gallium-67 chloride formed insoluble hydroxides and other gallium species in various buffer systems at physiologic pH; therefore, ^{67}Ga-citrate was chosen as the radiolabeling agent for the preparation of ^{67}Ga-fibrinogen.
3. A spectrophotometric assay was employed to determine if the observed binding capacity of deferoxamine was equivalent to the theoretical binding capacity. Results of this assay revealed that essentially all the deferoxamine was available for binding of metal cations.
4. Increasing aggregate formation of native fibrinogen with time was observed when a concentrated fibrinogen solution (6.8 mg/mL) was prepared and allowed to stand at room temperature over a 24 hour period. Therefore, the preparation, purification, and radiolabeling of deferoxamine-fibrinogen must be performed as quickly as possible to reduce protein denaturation. The coupling of deferoxamine to fibrinogen was performed within 1 hour after initial dissolution of fibrinogen in order to minimize denaturation of the protein.
5. Dialysis and gel filtration techniques were compared for their ability to remove unreacted deferoxamine and deferoxamine-glutaraldehyde from the desired deferoxamine-fibrinogen product. No statistically significant difference in final molar ratio, clottability, isotopic clottability, radiolabeling efficiency, and molecular weight was observed between the deferoxamine-fibrinogen products or between the radiolabeled preparations obtained with either of these two techniques. This indicates that both purification methods were similar

in their ability to remove unreacted reagents from deferoxamine-fibrinogen. However, due to the risk of aggregate formation of fibrinogen with time, as observed with Bio-Gel A-1.5m column chromatography, gel filtration using Bio-Gel P-6DG was considered to be superior to dialysis because of its speed in purifying the chelate-protein complex.

6. The influence of varying deferoxamine-fibrinogen molar ratio on the clottability of deferoxamine-fibrinogen and on the radiolabeling efficiency of ^{67}Ga-fibrinogen was investigated. A mean protein clottability of 75.4% and mean isotopic clottability of 79.2% were obtained with a mean final deferoxamine-fibrinogen molar ratio of 2.4:1. However, at this level of conjugation a radiolabeling efficiency of only 2.5% was attained. Increasing the number of deferoxamine molecules linked with fibrinogen to 7.1:1 increased the yield of ^{67}Ga-fibrinogen to 42.3% at the expense of reduced protein clottability and isotopic clottability. At higher molar ratios, an increased amount of protein polymerization was observed. The presence of free glutaraldehyde may have contributed to such polymerization. In addition, at higher molar ratios a 40 mL bed volume Bio-Gel P-6DG column was unsatisfactory in removing unreacted deferoxamine from the protein complex resulting in a decline in radiolabeling efficiency. Increasing the column bed volume to 65 mL allowed these complexes to be adequately purified although (i) it remained difficult to completely separate free deferoxamine from deferoxamine-fibrinogen, and (ii) a marked increase in protein polymerization was observed.
7. A mean final conjugation level of 7.1 moles of deferoxamine per mole of fibrinogen was considered to be the optimum molar ratio for the preparation of ^{67}Ga-fibrinogen. At this ratio, protein clottability averaged 62.6%, with an isotopic clottability of 70.2%, and a radiolabeling efficiency of 42.3%. Also, very little polymerization/aggregation of fibrinogen was observed at this level of deferoxamine-fibrinogen conjugation.
8. Increasing the fibrinogen concentration from 6.8 mg/mL to 10.2 mg/mL during the reaction, while maintaining a constant deferoxamine-fibrinogen ratio, significantly decreased radiolabeling efficiency and protein clottability. The final protein concentration, after purification of the complex, was also noticeably lower due to precipitation of

fibrinogen aggregates. Isotopic clottability was also found to decrease although this decrease was not statistically significant. The decrease in ^{67}Ga-fibrinogen yield and in isotopic clottability may be due to the unavailability of deferoxamine for binding radiogallium because of steric hindrance caused by the surrounding large number of fibrinogen molecules. Alternately, protein-protein coupling, caused by an increased amount of glutaraldehyde in the reaction mixture may result in the deferoxamine molecule becoming buried within the cross-linked molecule, again making it inaccessible for binding with ^{67}Ga. Intermolecular cross-linking would also result in aggregation and precipitation of the fibrinogen polymer thus lowering the protein concentration.

9. Twenty-four hour stability studies of purified ^{67}Ga-fibrinogen (2 to 3 MBq/mg) indicated that no free radioactivity could be detected in the ^{67}Ga-fibrinogen product.

10. No statistically significant difference between the specific activities of ^{67}Ga-fibrinogen was observed when the amount of ^{67}Ga-citrate, incubated with a constant amount of deferoxamine-fibrinogen, was doubled. A mean specific activity of 2.08 MBq/mg of ^{67}Ga-fibrinogen was achieved with 40 MBq of radioactivity versus 3.02 MBq/mg with 20 MBq ^{67}Ga-citrate. Due to the low specific activity attained, the two-step glutaraldehyde method was not considered to be practical for the development of ^{67}Ga-fibrinogen as a diagnostic agent of deep vein thrombosis.

11. Zinc metal contamination, ranging from approximately 19 nmoles/mL to greater than 3 μmoles/mL, was detected in all ^{67}Ga-citrate samples analyzed by neutron activation analysis. High specific activity radiolabeling of deferoxamine-fibrinogen with such solutions of ^{67}Ga may not be possible due to competition of zinc ions with ^{67}Ga for binding sites. Thus, trace metal contamination may contribute to the low radiolabeling efficiencies and specific activities observed.

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